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PRINCIPAL INVESTIGATOR: Allan I. Levey, M.D., Ph.D.

CONTRACTING ORGANIZATION: Emory University
Atlanta, Georgia 30322

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6. AUTHOR(S)
Allan I. Levey, M.D., Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Emory University
Atlanta, Georgia 30322

E-MAIL:
alevey@emory.edu

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13. ABSTRACT ***(Maximum 200 Words)***

The symptoms of Parkinson's disease (PD) are associated with a loss of substantia nigra dopaminergic neurons involved in modulation of function of the basal ganglia (BG). This report describes our progress in understanding the role of metabotropic glutamate receptors (mGluRs) as a novel target for the treatment of PD. Specifically, our aims are to localize mGluR4 in rat and monkey basal ganglia structures, to determine the role of this receptor vs. other subtypes in mediating the electrophysiological effects of glutamate in rat brain slices, and to determine the efficacy of drugs aimed mGluRs in relieving motor symptoms in hemi-parkinsonian monkeys. We found that group III mGluRs are presynaptic on striatal-pallidal terminals and that they mediate a reduction in IPSC amplitude in the SNr. Group III mGluRs also presynaptically inhibit EPSCs at the STN-SNr synapse. Group II mGluRs were found to mediate a presynaptic reduction of EPSCs in the SNr and group II agonist LY354740 reverses catalepsy in an animal model of PD. Furthermore, as these studies progress, the distribution and physiological roles of mGluRs in the BG raises the possibility that these receptors may provide targets for novel therapeutic agents for treatment of PD and related disorders.

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INTRODUCTON

Parkinson's disease (PD) is a common neurodegenerative disorder characterized by disabling motor impairments including tremor, rigidity, and bradykinesia. In PD there is a significant loss of nigrostriatal dopamine neurons that results in a series of neurophysiological changes that lead to a pathological excitation of the subthalamic nucleus (STN). The increased activity of STN neurons leads to an increase in glutamate release at STN synapses onto GABAergic projection neurons in the internal segment of the globus pallidus (GPi) and the substantia nigra pars reticulata (SNr). This glutamate-mediated over excitation of the BG output nuclei ultimately leads to a "shutdown" of thalamocortical projections and produces the motor impairments characteristic of PD[1]. Unfortunately, as the disease progresses, the efficacy of traditional dopamine replacement therapy becomes severly diminished, and severe motor and psychiatric side effects can[2]. Because of this, a great deal of effort has been focused on developing new approaches for the treatment of PD. In these studies we are pursuing a novel therapeutic approach by targeting drugs acting at metabotropic glutamate receptors (mGluRs). Eight mGluR subtypes have been cloned (designated mGluR1-mGluR8) from mammalian brain. Each subtype is classified in one of three classes (I-III). The present research focuses on group III mGluRs, in particular mGluR4. We aim to develop and characterize antibodies to mGluR4 which will be used to generate a map of receptor distribution in the basal ganglia of rat and rhesus monkey. We will determine the effect of selective group III mGluR agonists and antagonists on synaptic transmission at the major excitatory synapses in the SNr and GPi. Finally, we will evaluate the therapeutic potential of group III agonists in hemi-parkinosnian monkeys. Since mGluRs play an important role in the modulation of BG function, we expect that the results of this study may provide valuable insight into alternative treatment options for PD.

BODY

Specific Aim I.

To localize mGluR4a and 4b receptors in rat and monkey basal ganglia by immunohistochemical techniques using subtype- and isoform- specific antibodies.

This aim is focused on the development and characterization of antibodies against mGluR4a and mGluR4b. The antibodies are used as a tool to reveal the anatomical distribution of these receptors in the basal ganglia of rat and rhesus monkey. We have successfully developed and characterized a polyclonal antibody to the carboxy terminal portion of mGluR4a. An immunoblot (Figure 1) containing membrane protein from cells over-expressing mGluR2, mGluR5, mGluR7a, GluR4a, mGluR4b, and non-transfected Sf9 cells was probed with the affinity-purified mGluR4a antibody. Chemiluminescence revealed a dark band at the predicted molecular weight for mGluR4a (~100 kD). Furthermore, it shows the exclusive recognition of membrane protein from cells expressing mGluR4a. Imunocytochemistry (ICC) was then used to determine the distribution and localization of mGluR4a in the rat brain. With 3,3'-diaminobenzidine (DAB) as the chromagen, light microscopy shows a distinct pattern of mGluR4a distribution in the basal ganglia (Figure 2). Very low levels of mGluR4a were detected in the striatum (STR). Virtually no staining was detected in the substantia nigra pars compacta (SNc). The substantia nigra pars reticulata (SNr), entopeduncular nucleus (EPN), and globus pallidus (GP), however exhibited densely labeled neuronal fibers. Staining was absent when primary antibody was omitted, and when the antibody was pre-absorbed to mGluR4a synthetic peptide prior to the ICC incubation. Figure 3 is a high magnification photograph showing double labeling with mGluR4a antibodies and the presynaptic marker SV2. mGluR4a and SV2 immunoreactivity are clearly colocalized (arrows) along the outside of dendrites in the GP, suggesting a presynaptic localization of mGluR4a in the GP. Electron microscopy (Figure 4) was used to confirm the localization of this receptor to presynaptic terminals.

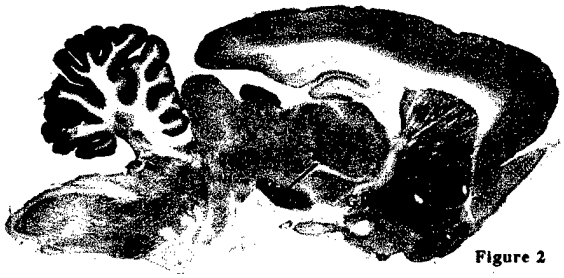


Figure 1

Figure 2


Figure 3

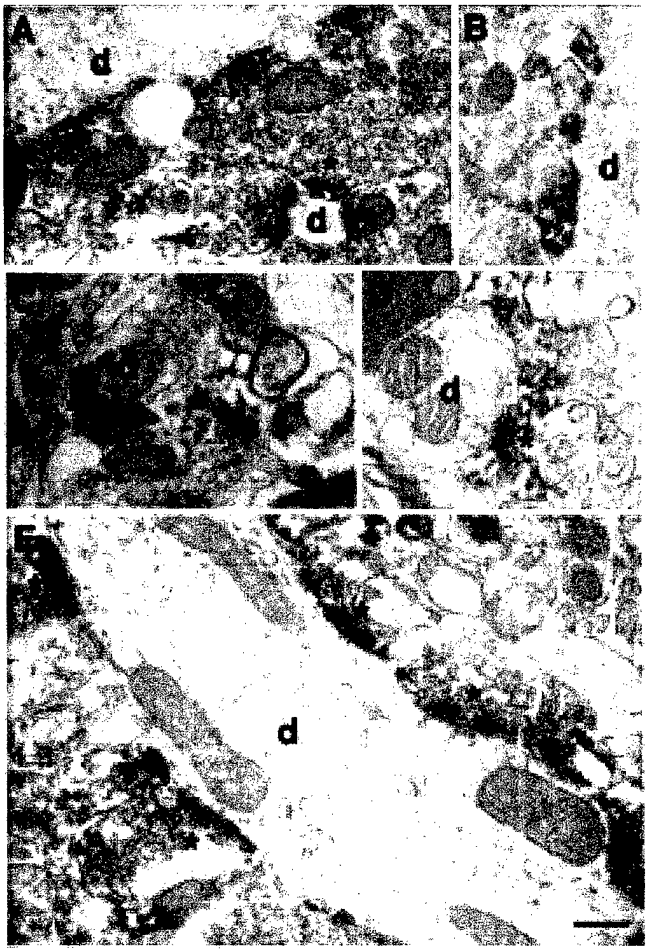
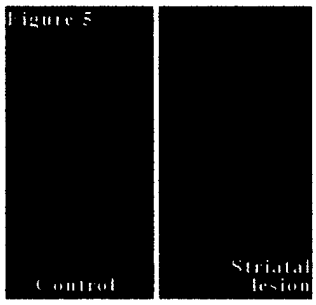


Figure 4. A-E: Electron micrographs demonstrating presynaptic mGluR4a immunoreactivity in the GP. Examples of mGluR4a axon terminals (asterisks) synapsing with dendrites (d) of cells in the GP. Scale bar = 400 nm in A-D; 350 nm in E.

Furthermore, quinolinate lesioning of the projecting neurons from the striatum to the GP induced a marked decrease in the ipsilateral but not contralateral (control) GP (Figure 5; red represents mGluR4a immunoreactivity). This suggests that such presynaptic localization is on striatopallidal terminals (Figure 5)[3]. Future experiments will employ immunocytochemistry to explore the effects of mGluR4a receptor expression and distribution in the basal ganglia of hemiparkinsonian rhesus monkeys that have been treated with mGluR agonists. Such experiments will be completed over the course of the next 3 years as the monkeys are sacrificed upon the completion of specific aim 3. In addition, all of the same characterization and localization experiments will be performed for mGluR4b, the other isoform.



Specific Aim II.

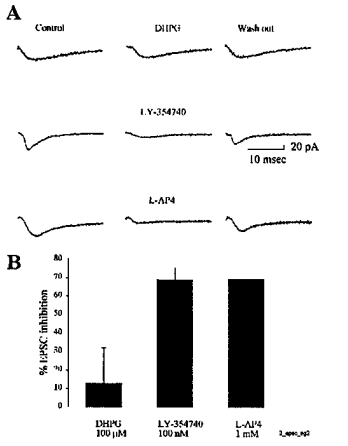
Determine the effect of selective group III mGluR agonists and antagonists on synaptic transmission at the major excitatory synapses In the output nuclei of the basal ganglia.

In this aim we are using whole cell patch clamp techniques to record the effects of mGluR agonists and antagonists on synaptic transmission in the BG. Over the course of the next three years this work will eventually provide a thorough characterization of the roles that mGluRs play at the major synapses of the BG. Up until now we have primarily focused our efforts on the SNr, where we have shown (Figure 7) that group III mGluRs mediate a presynaptic inhibition of EPSCs at the STN-SNr synapse [4].

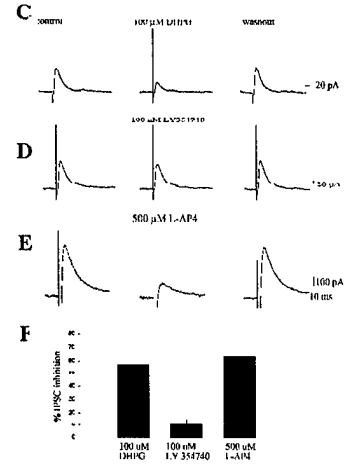
In addition, group III mGluRs mediate a reduction in IPSC amplitude in the[5]. To fully understand the role of mGluR4, however, we have been also carefully assessing the roles of other mGluR subtypes since glutamate will activate all receptors present in basal ganglia structures. Group II mGluRs were found to mediate a presynaptic reduction of EPSCs in the SNr. Consistent with this finding is the observation that the highly selective

group II agonist LY354740 reverses catalepsy in an animal model of PD [4, 6, 7]. Activation of group I mGluRs produces a reduction in IPSC amplitude and directly excites SNr projection neurons [5, 8, 9]. These findings suggest that activation of group I mGluRs can excite GABAergic projection neurons both by direct stimulation and by disinhibition. Current work is aimed at a more thorough characterization of the pharmacology of this effect, and at determining the pre- or post-synaptic locus of this synaptic inhibition. In addition, we have examined the direct effects of group I agonists on STN neurons and found that group I mGluRs, mGluR5, in particular mediates the depolarization of STN neurons[10]. Currently, biophysical studies of the reduction in IPSCs directed at determining the pre- versus post-synaptic loci of the groups III and I mGluR effects are being performed. Additional studies over the next three years will provide a complete characterization of the electrophysiological properties of mGluRs in the STN and the EPN (primate GPi). The ability of group II and group III mGluRs to modulate the excitatory transmission of glutamate at the STN-SNr synapse, combined with the data obtained on the ability of group I mGluRs to excite BG output neurons indicates that the development of novel therapeutic agents that target the specific receptors at these synapses could provide relief from the symptoms of PD.

Figure 7.



EPSCs Stimulation of the STN evokes a glutamatergic EPSC in SNr neurons, which is modulated by activation of mGluRs. (A) Bath application of the group II selective agonist LY354740 (100 nM), or the group III selective agonist L-AP4 (C), inhibits evoked EPSCs. The group I selective agonist DHPG (500µM) had no effect. The non-NMDA glutamate receptor antagonist CNQX (20 µM) completely blocked these currents indicating that they are glutamatergic EPSCs (data not shown) (B) Mean data demonstrating both group II and group III selective agonists inhibit synaptic transmission at the STN-SNr synapse. Data from representative neurons (A), or the mean + s.e.m. of data from 5 cells (B).



IPSPs Stimulation of inhibitory inputs evokes a GABAergic IPSC in SNr neurons, which is modulated by activation of mGluRs. (C) Bath application of the group I selective agonist DHPG (100 µM), or the group III selective agonist L-AP4 (500 µ M) inhibits evoked IPSCs (E). The group II selective agonist inhibits synaptic transmission at GABAergic synapses on SNr neurons (D). Data from representative neurons (C), (D), and (E) or the mean + s.e.m. of data from 5 cells(F).

Specific Aim III.

To evaluate the therapeutic potential of group III agonists in hemiparkinsonian monkeys.

This aim directly measures the efficacy of mGluR agonists on hemiparkinsonian monkeys. Two rhesus monkeys were behaviorally conditioned to tolerate transfers from their home cages into the primate chair. We then carried out base line observations using an automated activity monitoring cage with eight sets of infrared beams. Activity is being measured by counting infrared (IR) beam crossings over a 20-minute period. The pattern of IR crossings is also stored on computer disk, and can be used to later analyze behavioral patterns such as rotational behaviors, the amount of time spent in the upright posture, etc. In addition, we used a computer-assisted behavioral observation method, by which an observer scores movements of individual limbs of the animal by pressing keys on a computer keyboard. A computer stores the timing and length of key presses. More than one key can be scored at any given time. For assessment of more distal motor control, a Kluever-board technique was used for both upper extremities separately. For this, the monkey was placed in front of a plexi -glass board with 16 wells, into which raisins were placed. The time needed to empty all wells (or the number of wells emptied in 20 seconds) was scored. Finally, a behavioral rating scale was completed on each experimental day, scoring the presence or absence of parkinsonian motor signs, and dyskinetic movements/stereotypes. After the initial behavioral observations, the monkeys were each treated with a single injection of MPTP (0.4 mg/kg) into their right internal carotid artery, following published protocols[11]). Both animals have been observed after the injections, and a stable parkinsonian state has been documented with the above-mentioned behavioral observation methods in one animal. As discussed in the original proposal, we waited four months to make sure the animals do not have any further degree of spontaneous recovery. One animal has achieved a stable nigrostriatal lesion and is scheduled to receive steel recording chambers directed at the GPi and SNr to carry out intracerebral injections into these areas. The second animal has partially recovered from the MPTP treatment, and will need additional MPTP injections in order to induce a more persistent hemiparkinsonian state.

Additionally, since previous studies have shown that the most potent group III agonist, L-AP4, exhibits some affinity for the NMDA receptor[12], we are in the process of performing preliminary in vivo studies to assess the effects of NMDA-mediated excitotoxicity of group III mGluR agonists in the SNr of rats. Stereotaxic coordinates are used to map the precise location of the SNr and the drug is administered by direct injection into the SNr. Immunocytochemistry will be used to examine the effects of group III mGluR receptor distribution and expression after application of such drugs. The data obtained from these studies will ensure that LAP-4 is safe and nontoxic to brain tissue prior to administering this agonist to primates.

KEY RESEARCH ACCOMPLISHMENTS

- Antibodies to mGluR4a were developed, fully characterized, and used to describe the anatomical distribution and localization of mGluR4a in rat brain.

- Whole cell patch clamp techniques were used to show that:
  1. Group III mGluRs mediate a presynaptic inhibition of EPSCs at the STN-SNr synapse.
  2. Group III mGluRs mediate a reduction in IPSC amplitude in the SNr.
  3. Group II mGluRs mediate a presynaptic reduction of EPSCs in the SNr.
  4. Group II agonist LY354740 reverses catalepsy in an animal model of PD.
  5. Activation of group I mGluRs produces a reduction in IPSC amplitude and directly excites SNr projection neurons.
  6. Group I mGluRs, mGluR5, in particular mediate the depolarization of STN neurons.

- One rhesus monkey has achieved a stable hemiparkinsonian state, was behaviorally trained to tolerate transfers from its home cage into a primate chair, and was trained in food retrieval tasks.

REPORTABLE OUTCOMES

Papers

Bradley, S.R., *et al.*, *Immunohistochemical localization of subtype 4a metabotropic glutamate receptors in the rat and mouse basal ganglia.* J Comp Neurol, 1999. **407**(1): p. 33-46.

Manuscripts

1. Bradley, S.R., *et al.*, *Activation of Group II metabotropic glutamate receptors inhibits synaptic excitation of the substantia nigra pars reticulata.* (Submitted), 1999.

2. Marino, M., *et al.*, *Activation of the Group I metabotropic glutamate receptor mGluR1 produces a direct excitation and mediates a slow EPSP in the substantia nigra pars reticulata.* (In Preparation), 1999.

Abstracts

1. Awad, H. and P. Conn, *Physiological actions of metabotropic glutamate receptors in neurons of the subthalamic nucleus.* Neuropharmacology Abstr., 1999. **38**(10): p. A3.
2. Bradley, S.R., *et al.*, *Physiological roles of presynaptically localized type 2, 3 and 7 metabotropic glutamate receptors in rat basal ganglia.* Soc. Neurosci. Abstr., 1999. **25**(Abstr. No. 176.16): p. 446.

3. Wittmann, M., *et al.*, *GABAergic inhibition of rat substantia nigra pars reticulata projection neurons is modulated by metabotropic glutamate receptors.* Soc. Neurosci. Abstr., 1999. **25**(Abstr. No. 176.18): p. 446.

4. Marino, M., *et al.*, *Potential antiparkinsonian actions on metabotropic glutamate receptors in the substantia nigra pars reticulata.* Neuropharmacology Abstr., 1999. **38**(10): p. A29.

5. Marino, M., *et al.*, *Direct excitation of gabaergic projection neurons of the rat substantia nigra pars reticulata by activation of the mGluR1 metabotropic glutamate receptor.* Soc. Neurosci. Abstr., 1999. **25**(Abstr. No. 176.17): p. 446.

CONCLUSIONS

Western blot analysis revealed that mGluR4a antibodies selectively and specifically recognize mGluR4a. With these antibodies we have shown that low levels of mGluR4a were found in the STR, SNr, and EPN, key basal ganglia structures involved in the manifestation of Parkinsonian symptoms. Virtually no staining was detected in the SNc. The globus pallidus GP contains a high concentration of presynaptic mGluR4a neuronal fibers that project from the striatum. The relative high concentration of presynaptic straito-pallidal mGluR4a terminals is significant because it provides physical evidence in support of the hypothesis that mGluR4a can serve a presynaptic heteroreceptor involved in regulating GABA release [13-15]. In the case of our studies, the regulation of GABA release by mGluR4a would occur at striatopallidal and striato-SNr terminals [3] and provide a potential target for the treatment of PD.

The electrophysiological studies showed that group III mGluR agonists mediate a presynaptic inhibition of EPSCs at the STN-SNr synapse and a reduction in IPSC amplitude in the SNr. Group II mGluRs mediate a presynaptic reduction of EPSCs in the SNr. The Group II agonist LY354740 reverses catalepsy in an animal model of PD. Activation of Group I mGluRs produces a reduction in IPSC amplitude and directly excites SNr projection neurons. The ability of group II and group III mGluRs to modulate the excitatory transmission of glutamate at the STN-SNr synapse, combined with the data obtained on the ability of group I mGluRs to excite BG output neurons and the anatomical findings all provide compelling evidence that the development of novel therapeutic agents that target the specific receptors at these synapses could provide relief from the symptoms of PD. Continuing studies will be essential to further define the roles of mGluR4 and related mGluRs in basal ganglia function, and most critically, their value as targets for novel therapeutic drugs in hemi-parkinsonian monkeys.

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4. Bradley, S.R., *et al.*, *Physiological roles of presynaptically localized type 2, 3 and 7 metabotropic glutamate receptors in rat basal ganglia.* Soc. Neurosci. Abstr., 1999. **25**(Abstr. No. 176.16): p. 446.
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APPENDICES

I. Bradley, S.R., *et al., Immunohistochemical localization of subtype 4a metabotropic glutamate receptors in the rat and mouse basal ganglia.* J Comp Neurol, 1999. **407**(1): p. 33-46.

II. Bradley, S.R., *et al., Activation of Group II metabotropic glutamate receptors inhibits synaptic excitation of the substantia nigra pars reticulata.* (Submitted), 1999.

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Immunohistochemical Localization of Subtype 4a Metabotropic Glutamate Receptors in the Rat and Mouse Basal Ganglia

STEFANIA RISSO BRADLEY,[1] DAVID G. STANDAERT,[2] KENNETH J. RHODES,[3] HOWARD D. REES,[4] CLAUDIA M. TESTA,[2] ALLAN I. LEVEY,[4] AND P. JEFFREY CONN[1]*

[1]Department of Pharmacology, Emory University School of Medicine, Atlanta, Georgia 30322
[2]Neurology Service, Massachusetts General Hospital and Harvard Medical School, Boston, Massachusetts 02114
[3]CNS Disorders, Wyeth-Ayerst Research, Princeton, New Jersey 08543
[4]Department of Neurology, Emory University School of Medicine, Atlanta, Georgia 30322

ABSTRACT

Recent studies suggest that metabotropic glutamate receptors (mGluRs) may play a significant role in regulating basal ganglia functions. In this study, we investigated the localization of mGluR4a protein in the mouse and rat basal ganglia. Polyclonal antibodies that specifically react with the metabotropic glutamate receptor subtype mGluR4a were produced and characterized by Western blot analysis. These antibodies recognized a native protein in wild-type mouse brain with a molecular weight similar to the molecular weight of the band from a mGluR4a-transfected cell line. The immunoreactivity was absent in brains of knockout mice deficient in mGluR4. mGluR4a immunoreactivity was most intense in the molecular layer of the cerebellum. We also found a striking mGluR4a immunoreactivity in globus pallidus, and moderate staining in substantia nigra pars reticulata and entopeduncular nucleus. Moderate to low mGluR4a immunoreactivity was present in striatum and other brain regions, including hippocampus, neocortex, and thalamus. Double labeling with mGluR4a antibodies and antibodies to either a dendritic marker or a marker of presynaptic terminals suggest a localization of mGluR4a on presynaptic terminals. Immunocytochemistry at electron microscopy level confirmed these results, revealing that in the globus pallidus, mGluR4a is mainly localized in presynaptic sites in axonal elements. Finally, quinolinic acid lesion of striatal projection neurons decreased mGluR4a immunoreactivity in globus pallidus, suggesting a localization of mGluR4a on striatopallidal terminals. These data support the hypothesis that mGluR4a serves as a presynaptic heteroreceptor in the globus pallidus, where it may play an important role in regulating g-amino-n-butyric acid (GABA) release from striatopallidal terminals. J. Comp. Neurol. 407:33–46, 1999.

Indexing terms: globus pallidus; corticostriatal pathway; presynaptic heteroreceptor; electron microscopy; confocal microscopy

Parkinson's disease (PD) is a common basal ganglia (BG) neurodegenerative disorder resulting in disabling motor impairment (tremor, rigidity, and bradykinesia). Loss of nigrostriatal dopamine neurons results in a series of neurophysiological changes that lead to overactivity of the globus pallidus (GP: one of the output nuclei of the BG) and consequent "shutdown" of thalamocortical structures to produce the motor symptoms (Ciliax et al., 1997; Wichmann and Delong, 1997). Although therapies have traditionally utilized dopamine replacement strategies, this

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*Correspondence to: Dr. P. Jeffrey Conn, Dept. of Pharmacology, Rollins Research Building, Emory University School of Medicine, Atlanta, GA 30322. E-mail: PCONN@pharm.emory.edu

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approach eventually fails in most patients. Exciting advances in understanding of the pathological changes in BG circuitry in PD patients coupled with recent findings suggesting abundant localization of metabotropic glutamate receptors (mGluRs) in key BG nuclei suggest that mGluRs could provide novel targets for therapeutic agents designed to treat PD patients. In the present study, we examined the localization of a mGluR subtype protein, mGluR4a, at crucial sites within BG circuits.

Glutamate is the principal excitatory neurotransmitter in the brain, and is present at many synapses along the BG circuits. It is now clear that the physiological effects of glutamate are mediated by ligand-gated cation channels, known as ionotropic glutamate receptors (iGluRs), and by G-protein-linked receptors, referred to as mGluRs. By activating mGluRs, glutamate can modulate transmission and neuronal excitability at the same synapses at which it elicits fast excitatory synaptic responses (see Conn et al., 1995; Conn and Pin, 1997, for reviews). To date, eight mGluR subtypes have been identified by molecular cloning and these receptors can be placed into three groups based on sequence homology, coupling to second messenger systems, and pharmacological profiles (see Conn and Pin, 1997, for review). Group I mGluRs include mGluR1 and mGluR5, which couple primarily to increases in phosphoinositide hydrolysis in expression systems. The group II mGluRs (mGluR2 and mGluR3), and the group III mGluRs (mGluR4, mGluR6, mGluR7, and mGluR8), all couple to inhibition of cAMP production in expression systems.

Previous studies suggest that presynaptic group II and group III mGluRs play important roles in regulating excitatory (Lovinger, 1991; Lovinger et al., 1993; Lovinger and McCool, 1995; Pisani et al., 1997) and inhibitory (Calabresi et al., 1992, 1993; Stefani et al., 1994) transmission in the striatum (STR) and that postsynaptic group I mGluRs regulate striatal cell excitability and N-methyl-D-aspartic acid (NMDA) receptor currents (Calabresi et al., 1992; Colwell and Levine, 1994; Pisani et al., 1997). Moreover, intrastriatal injection of mGluR agonists induces rotational behavior (Sacaan et al., 1991, 1992; Kaatz and Albin, 1995; Kearney et al., 1997) and this effect is abolished by lesions of the subthalamic nucleus (Kaatz and Albin, 1995).

Despite these advances in our understanding of the roles of mGluRs in STR, little is known about the roles of mGluRs in regulating the function of other BG structures. However, in recent years, we and others have used in situ hybridization and immunocytochemistry techniques to show that specific mGluR subtypes are richly distributed in BG structures where they are differentially localized at specific pre- and postsynaptic sites. For instance, group I mGluRs, mGluR1 and mGluR5, are expressed in the substantia nigra pars reticulata (SNpr) and entopeduncular nucleus (ET) neurons (Testa et al., 1994) and mGluR1a immunoreactivity is abundant along the surface of microtubule-associated protein 2 (MAP2)-immunoreactive dendritic processes in GP and SNpr (Testa et al., 1998). Furthermore, in situ hybridization studies suggest that group II mGluR subtype mGluR2 is moderately abundantly expressed in neurons of the subthalamic nucleus (STN; Testa et al., 1994). Finally, both mGluR4 and mGluR7 (but not mGluR6 or mGluR8) mRNA are moderately abundantly expressed in STR (Nakajima et al., 1993; Testa et al., 1994; Saugstad et al., 1994; Duvoisin et al., 1995; Kosinski et al., 1999) and double-labeling in situ hybridization revealed that mGluR7 mRNA is in projection neurons (Kosinski et al., 1999). More recent immunocytochemistry studies show that mGluR7a is widely distributed in rat brain (Bradley et al., 1996, 1998; Shigemoto et al., 1996, 1997) and it is presynaptically localized on terminals of g-amino-n-butyric acid (GABA)ergic striatopallidal and striatonigral synapses (Kosinski et al., 1999).

The finding that mGluR4 is expressed in striatal neurons suggests that this mGluR could play an important role in regulating BG function. However, the precise localization of mGluR4 protein in BG is not known. To begin to dissect the distribution of the mGluR4a in BG, we have produced and characterized polyclonal antibodies highly specific for mGluR4a.

MATERIALS AND METHODS

Production and characterization of polyclonal antibodies that specifically interact with mGluR4a.

Antibodies were generated against synthetic peptides corresponding to the putative intracellular C-terminal domain of mGluR4a. Rabbit polyclonal antisera were prepared and affinity-purified as described previously (Bradley et al., 1996). These antibodies specifically recognized mGluR4a but not other mGluR subtypes or the other mGluR4 splice variant, mGluR4b, as shown below. In double labeling experiments, incubations were performed using combination of mGluR4a antibodies with one of the following: monoclonal antibodies to MAP2 (Sigma, St. Louis, MO; 1:1,000), Enkephalin (Enk; Chemicon Inc., Temecula, CA; 1:2,000); synaptic vesicle protein 2 (SV2; 1:10) obtained from Dr. K. Buckley, Harvard Medical School (Buckley and Kelly, 1985).

Immunoblot analysis

Spodoptera frugiperda, Sf9, insect cells transfected with mGluR4a or mGluR4b were generously supplied by Dr. David Hampson (University of Toronto, Canada). BHK cells stable transfected with mGluR1a were generously supplied by Betty Haldeman of Zymogenetics (Seattle, WA). BHK cells stable transfected with mGluR7a (Saugstad et al., 1994) were generously supplied by Dr. Thomas Segerson (Vollum Institute, Portland, OR). BHK cells transfected with mGluR2 were generously supplied by Dr. Christian Thomsen (Novo Nordisk), and HEK cells transfected with mGluR5 were kindly supplied by Dr. Carl Romano (Washington University School of Medicine, St. Louis, MO). Membranes prepared from transfected cells and from rat brain regions were used for immunoblot studies with the mGluR-directed antibodies. Adult male Sprague-Dawley rats were killed and microdissected brain regions were immediately homogenized with a Brinkman Polytron in 10 mM Tris and 1 mM EDTA (pH 7.4) containing 2 µg/ml of leupeptin, 2 µg/ml of aprotinin, 2 µg/ml of pepstatin A. The homogenates were centrifuged at 2,250 RPM for 5 minutes, the supernatants decanted and recentrifuged at 13,000 RPM for 30 minutes. The membrane pellets were resuspended, protein concentration determined, and membranes kept at -70°C until use. Membranes from rat brain or cell lines were subjected to sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE; 10% acrylamide) and transferred to PVDF membranes (Millipore, Bedford, MA) by electroblotting as described by Towbin et al. (1979). The blots were blocked

with 5% nonfat dry milk in Tris-buffered saline (TBS; 20 mM Tris-HCl in 1.5% NaCl; pH 7.4) at room temperature for 30 minutes. Blots were then incubated overnight at 4°C with affinity-purified mGluR antibodies (0.5 mg/ml) in TBS. Membranes were then rinsed and incubated for 30 minutes with peroxidase-conjugated goat anti-rabbit IgG (Bio-Rad, Richmond, CA) at room temperature. Following several washes in TBS, immunoreactive proteins were visualized with enhanced chemiluminescence (Amersham Pharmacia Biotech. Inc, Piscataway, NJ), as recommended by the manufacturer. For preadsorption experiments, antibodies were preadsorbed with 10 µg/ml of homologous peptide for 1 hour at room temperature.

Immunocytochemistry

Immunohistochemistry was performed using well established methods (Bradley et al., 1996). For examination at the light microscopic level, adult male Sprague-Dawley rats (n = 10) were anesthetized with 4% chloral hydrate and transcardially perfused with 3% paraformaldehyde followed by 10% sucrose (200–250 ml of each). The protocol used in these studies has been reviewed and approved by the Emory University Institutional Animal Care and Use Committee (IACUC). Rat brains were removed and cryoprotected in 30% sucrose at 4°C in 0.1 M phosphate buffer (pH 7.6; 48 hours at 4°C), frozen on dry ice, and sectioned at 40–50 µM on a freezing sliding microtome. Mouse brains (n = 6) were rapidly removed and fixed by immersion in 3% paraformaldehyde for 7 hours at 4°C, then incubated in 10% sucrose for 48 hours at 4°C and finally incubated in 30% sucrose for 48 hours at 4°C. Mouse brains were then frozen on dry ice and sectioned at 40–50 µM on a freezing sliding microtome. Sections were collected in 50 mM TBS (pH 7.2) at 4°C. Tissue sections through the entire brain were processed for immunocytochemistry. Sections were preblocked in TBS with 4% normal goat serum (NGS) and avidin (10 mg/ml) for 30 minutes and subsequently in TBS with 4% NGS and biotin (50 µg/ml) for another 30 minutes. The sections were then incubated with primary antibody (0.5 µg/ml) in TBS and 2% NGS over two nights at 4°C. The avidin-biotin peroxidase method (Vectastain Elite ABC kit; Vector Laboratories, Burlingame, CA) was used to detect mGluR4a immunoreactivity. The tissue was rinsed several times in TBS and the peroxidase reaction was developed in 0.05% DAB and 0.01% H_2O_2 for 10 minutes. Sections were finally rinsed in TBS and mounted on subbed slides. Sections were dehydrated in alcohols, defatted in xylene, and coverslipped for analysis.

Control experiments were performed in which sections were incubated in TBS without primary antibody. Further control experiments were performed in which primary antibody was preincubated for 30 minutes at room temperature with the homologous peptide (10 mg/ml).

For electron microscopy, Sprague-Dawley rats 250–300 g (n = 4) were deeply anesthetized with 4% chloral hydrate, then perfused transcardially with a phosphate-buffered solution of 3% paraformaldehyde and 0.1 % glutaraldehyde for 10 minutes (250 ml). Brains were postfixed for 1 hour at 4°C and then and sectioned at 40 µm using a vibrotome (Technical Products International, Inc., St. Louis, MO). Sections were collected in 0.1 M phosphate buffer then rinsed several times with TBS (10 minutes each rinse) before being processed as described for light microscopy. Following the treatment with 0.05% DAB and 0.01% H_2O_2 for 10–15 minutes, the sections were rinsed several times in TBS. Sections were then incubated overnight in 2% glutaraldehyde in 0.1 M phosphate buffer. After rinsing the sections twice for 10 minutes. in phosphate buffer and then in cacodylate buffer (0.1 M), they were postfixed in 1% osmium tetroxide in 0.1 M cacodylate buffer for 30 minutes. Slices were then rinsed twice for 10 minutes in 0.1 M cacodylate buffer, followed by a rinse in 0.05 M acetate buffer. Slices were block-stained overnight in aqueous 2% uranyl acetate, followed by a rinse in 0.05 M acetate buffer. The tissue was then dehydrated in graded ethanols and finally in propylene oxide for 5 minutes before leaving overnight in Epon 1:1 propylene oxide. The sections were finally embedded in Epon resin between glass slides and left at 60°C for 2 days. Blocks were dissected from the GP and mounted on stubs and sectioned using an ultramicrotome (RMC MT5000 or Reichert Ultracut). Ultrathin sections were collected on uncoated copper mesh grids for analysis with electron microscopy (H-7500, Hitachi).

Fluorescence double-label immunohistochemistry

Dual-label immunohistochemistry was conducted as described in Kosinski et al. (1997). Sections from Sprague-Dawley rat brains (n = 5) were washed in 0.1 M sodium phosphate buffer/saline, pH 7.4, containing 0.9% NaCl, incubated in 3% normal goat serum with 0.3% Triton X-100 in sodium phosphate buffer/saline, pH 7.4, and 0.1% sodium azide for 1 hour, and then incubated 48 hours at 4°C in a solution containing the primary antibodies to mGluR4a in combination with one of the monoclonal antibodies. Sections were then washed in phosphate-buffered saline (PBS) and incubated sequentially in two fluorescent secondary antibodies: the mGluR4a antibodies was visualized using a goat anti-rabbit antiserum coupled to indocarbocyanide (Cy3; 1:400; Jackson Labs., West Grove, PA) whereas Enk, the presynaptic marker SV2, and MAP2 staining were visualized with goat anti-mouse antiserum labeled with fluorescein isothiocynate (FITC) or Cy5 (both from Jackson Laboratories). The sections were then mounted onto gelatin-coated slides, dried, and coverslipped by using glycerol containing 100 mM Tris, pH 8.0, and 0.2% p-phenylenediamine (Sigma, St. Louis, MO) to retard fading. Each experiment included control tissue, processed with omission of one or both primary antibodies.

Preparations were examined using a BioRad Laser Confocal System (MRC 1000) equipped with a Leica DMBR microscope and an argon-krypton laser. High magnification images were obtained by illuminating the section with a single laser line and collecting the image using an appropriate emission filter: for Cy3, excitation at 488 nm and a 522 nm bandpass filter. For each wavelength, four sequential images 1024 × 1024 pixels in size with an 8-bit pixel depth were obtained and averaged, using a Kalman filtering method to reduce noise. Dual label images were obtained by collecting the separate images sequentially, and reconstructing the images in color using Adobe Photoshop software (Mountain View, CA).

Striatal quinolinic acid lesion

Quinolinic acid (100 nmol in 2 ml 0.1 M phosphate buffer, pH 7.4 , Sigma) was injected into the left anterior striatum of adult male Sprague-Dawley rats (Charles River; 200–250 g; n = 3) as described previously (Orlando et al., 1995). Animals were anesthetized with sodium

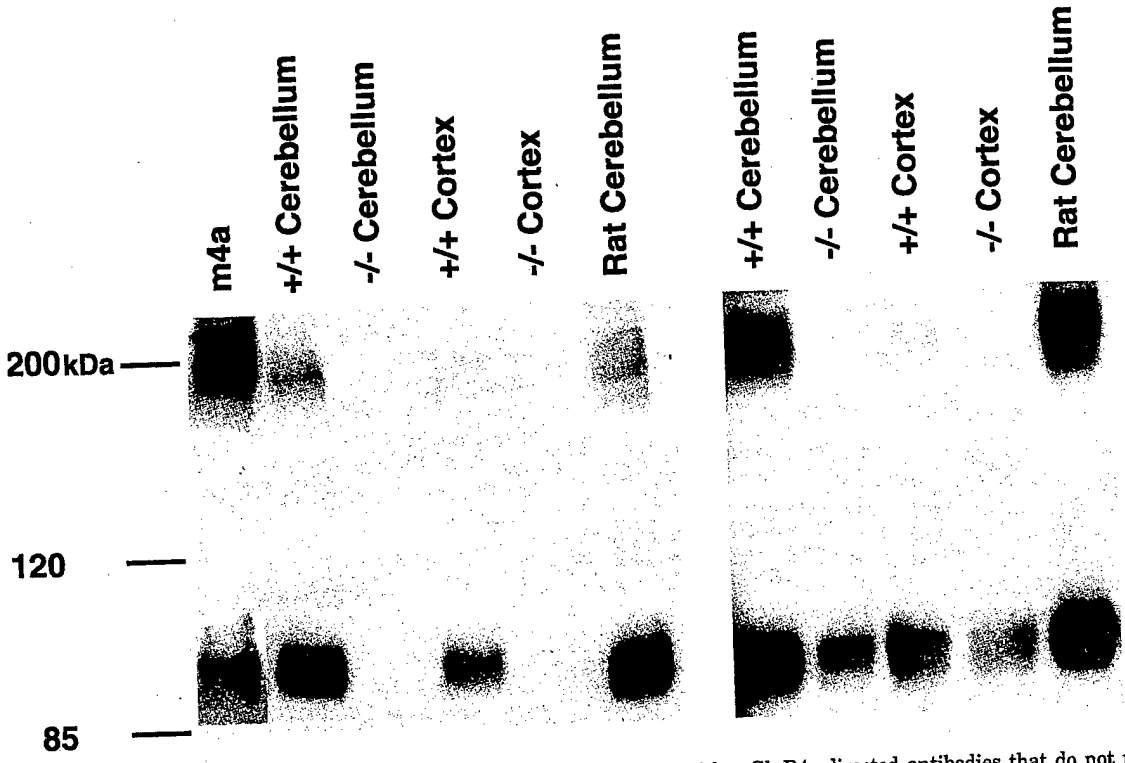


Fig. 1. Molecular specificity of metabotropic glutamate receptor (mGluR4a) antibodies. Western blot analysis was used with affinity-purifed polyclonal antibodies directed against mGluR4a from different rabbits to determine immunospecificity in membranes from wild type mouse (+/+) cerebellum and cortex, from mouse lacking mGluR4 (-/-; same regions), and from rat cerebellum. The left panel shows immunoreactivity with mGluR4a-directed antibodies that do not react with any proteins in mutant mouse, wheras the right panel shows a different elution of antibodies that recognize a protein in mutant mouse with identical molecular weight than mGluR4a. Molecular weight standards are designated on left in kilodaltons (kDa).

pentobarbital (Anpro Pharmaceutical) and surgery was performed in a stereotaxic apparatus using coordinates 2.6 mm lateral to and at the anteroposterior position of bregma, and 4.5 mm ventral to the dural surface (Paxinos and Watson, 1982). Injections were made over 3 minutes with a 10-ml Hamilton syringe fitted with a 26-gauge blunt-tipped needle. Transcardiac perfusion was performed 2 weeks after lesioning as described above. The extent of the lesions were evaluated on thionin-stained coronal sections through the striatum. Three brains in which the lesion was restricted to the anterior striatum and no damage was detected in the GP were chosen for the study.

RESULTS

Immunoblot analysis

Affinity-purified antibodies directed against mGluR4a were first characterized by Western blotting analysis with membranes (25 µg protein) from either wild type (WT) mouse brain or mouse brains from mutant mice lacking mGluR4 (Pekhletski et al., 1996). Figure 1 shows immunoblot analysis with polyclonal antibodies purified from two different rabbits. Consistent with our previous study (Bradley et al., 1996), both sets of antibodies reacted with a band of approximately 100 kDa in mouse and rat brain homogenates. This band directly corresponds with a band of the same molecular weight in cells expressing mGluR4a. In addition, both sets of antibodies react with a higher molecular weight band (approximately 200 kDa) that is likely to represent a dimer of mGluR4a (Romano et al., 1996; Bradley et al., 1996).

Analysis of immunoreactivity in mutant mice lacking mGluR4 revealed that one set of antibodies is highly specific for mGluR4a and does not react with proteins in brains from the mGluR4 knockout (KO) mice (Fig. 1, left). However, other antibodies cross-react with an unknown protein in tissue from mGluR4a KO mice (Fig. 1, right). Interestingly, the cross-reactive product present in mGluR4 KO mice had the same molecular weight as mGluR4a in cell lines and wild-type mice, making detection of the cross-reactivity impossible without the availability of the mutant mice. These findings suggest that previous immunocytochemistry studies using the latter antibody preparation likely reflects reactivity with both mGluR4a and the unidentified protein (Bradley et al., 1996).

The more specific antibodies that did not react with proteins in mGluR4 KO mice were used for further characterization to determine whether these antibodies are specific for mGluR4a relative to other mGluR subtypes. Western blot analysis was performed with membranes from control (untransfected) cells, and cell lines (5 µg protein) transfected with either mGluR5, mGluR2, mGluR4a, mGluR4b, or mGluR7a. Affinity-purified antibodies directed against mGluR4a reacted with a band at about 100 KDa in homogenates of cells transfected with the mGluR4a but not with homogenates of cells transfected with other mGluR subtypes (Fig. 2). The mobility of this protein was consistent with the predicted molecular

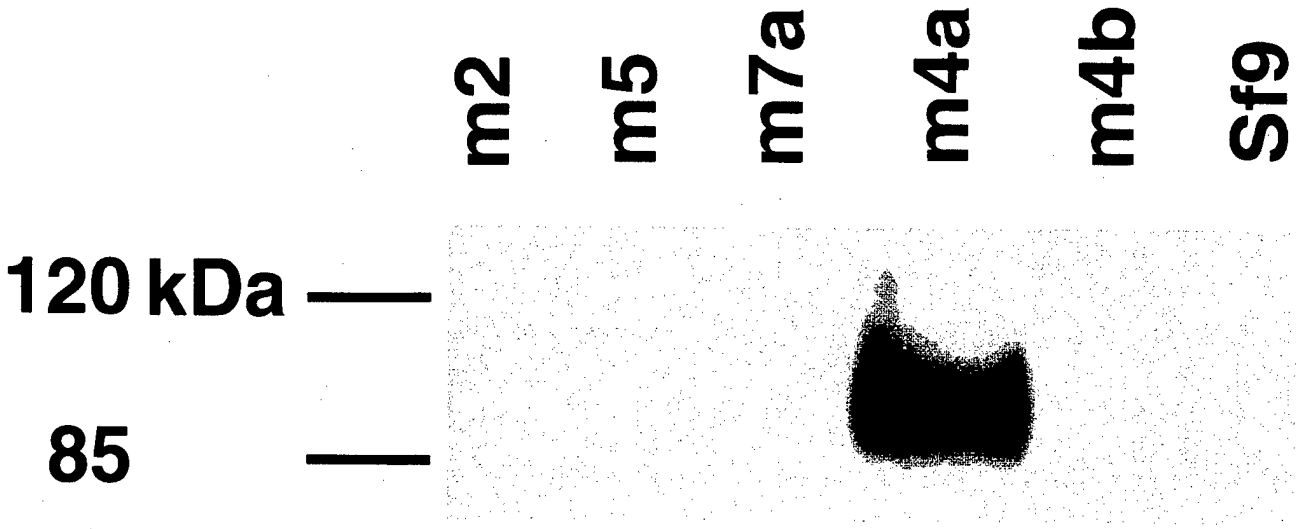


Fig. 2. Antibodies that specifically recognize metabotropic glutamate receptors (mGluR4a) in wild type mouse and nothing in mouse lacking mGluR4 do not cross-react with any other known mGluRs. Western blot analysis was used to determine the specificity of our antibodies for subtype 4a mGluRs. As shown in the figure, mGluR4a antibodies react with a band of about 100 kDa in homogenates from membranes of cell line transfected with mGluR4a, but mGluR4a immunoreactivity is absent in homogenates from cell lines transfected with mGluR2 (m2), mGluR5 (m5), mGluR7a (m7a), mGluR4b (m4b), or untransfected cell line (Sf9). Molecular weight standards are designated on left in kilodaltons.

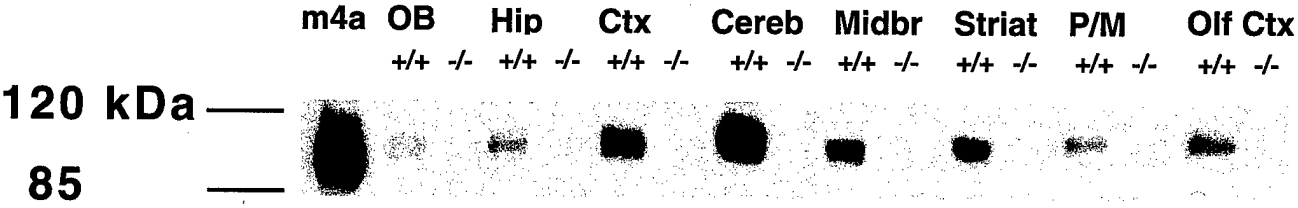


Fig. 3. Regional distribution of metabotropic glutamate receptor (mGluR4a) immunoreactivity in mouse brain. Western blot analysis was used to determine immunoreactivity with antibodies directed against mGluR4a in membranes (25 μg of protein) from several regions of wild type mouse (+/+) brain. Regions analyzed included neocortex (Ctx), cerebellum (Cereb), olfactory bulb (OB), piriform cortex/amygdala (Olf Ctx), pons/medulla (P/M), hippocampus (Hip), striatum (Striat), and midbrain (Midbr). These antibodies do not react with homogenates from membranes of mouse lacking mGluR4 (-/-).

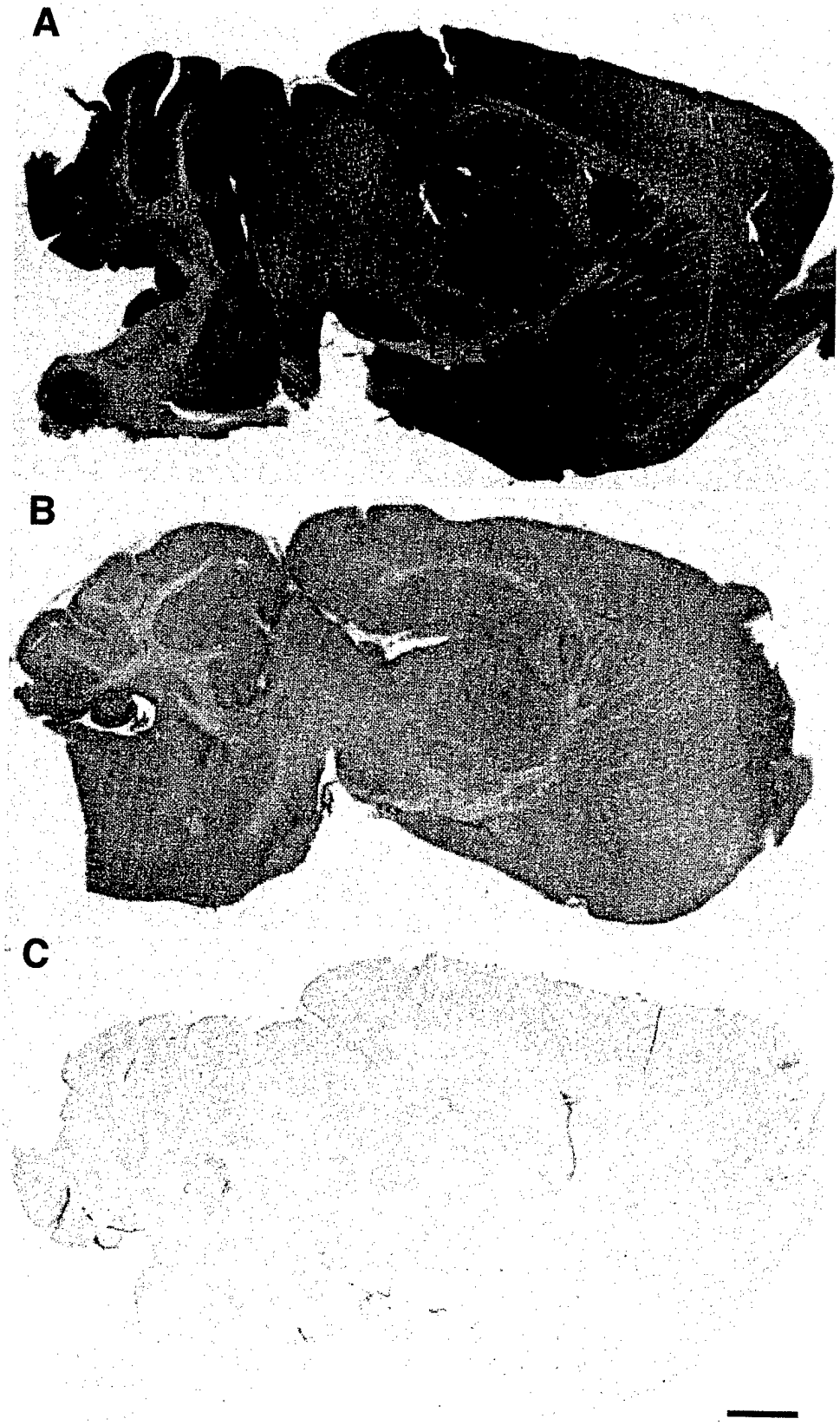
weight of mGluR4a based on its amino acid sequence. Preadsorption of mGluR4a antibody with homologous peptide totally abolished all immunoreactive bands (not shown).

The regional distribution of mGluR4a immunoreactivity was determined in a number of dissected brain regions from WT and mGluR4 KO mice (Fig. 3). Antibodies directed against mGluR4a reacted with different intensities in several brain regions in WT mice. The strongest immunoreactivity was observed in the cerebellum. Immunoreactivity was also present in the cortex, striatum, olfactory cortex, and midbrain. Relatively weak immunoreactivity was detectable in the hippocampus, olfactory bulb, and pons/medulla. Immunoreactive bands were not detected in any brain region from mGluR4a KO mice.

Immunocytochemical distribution of mGluR4a in mouse and rat brain

Affinity-purified antibodies that do not react with proteins in brains from the mGluR4 KO mice were used for immunocytochemical localization of mGluR4a in mouse and rat brain. Immunocytochemistry revealed specific staining with antibodies directed against mGluR4a in several brain regions in wild type mouse brain (Fig. 4A), whereas immunoreactivity was absent in brains from mice lacking mGluR4 (Fig. 4B). Immunoreactivity in WT mice was virtually abolished when the affinity-purified antibodies were preadsorbed with the homologous peptide (Fig. 4C). The same immunoreactive pattern was found in rat brain (not shown). Taken together with the high specificity of the antibodies demonstrated in the Western blot analysis, these data suggest that staining with these antibodies is highly selective for mGluR4a.

In the forebrain, mGluR4a showed a striking predilection for BG structures. Confocal fluorescence microscopy showed intense mGluR4a immunoreactivity in fibers in the GP (Fig. 4A). The STR overall exhibited very low intensity staining. Occasionally large labeled fibers were observed traversing the border of the STR with the GP (Fig. 4A). Only very low intensity was seen in the SNpr, and the large neurons of the substantia nigra pars compacta (SNpc) were not detectably labeled (Fig. 4A). In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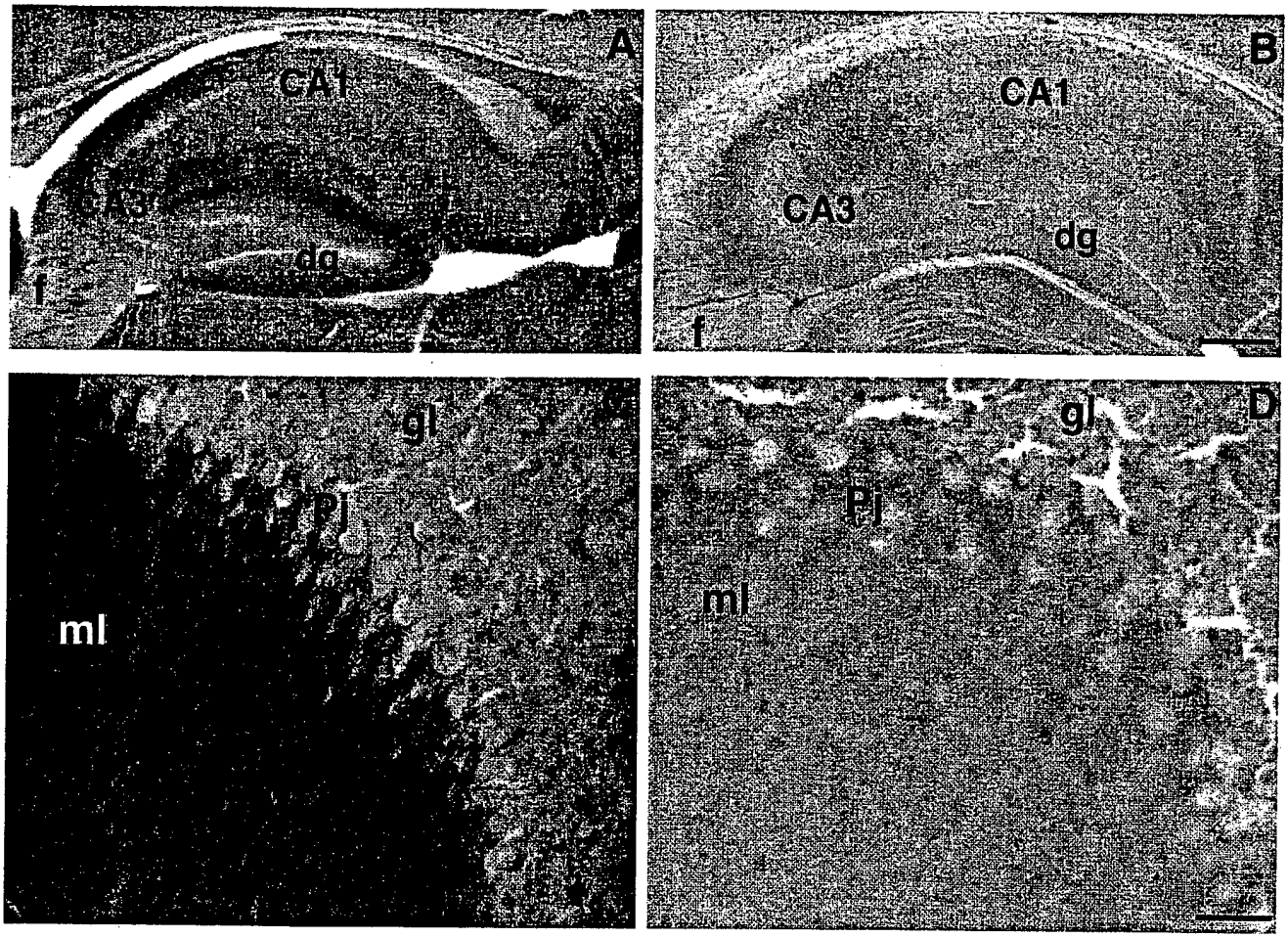

Fig. 5. Immunocytochemical staining of a section through the hippocampus and cerebellum in wild type and mutant mouse. Staining with antibodies directed against metabotropic glutamate receptor (mGluR4a) is shown in the hippocampus of wild type mouse (**A**) and mouse lacking mGluR4 (**B**). In wild type mouse, mGluR4a immunoreactivity was present in the middle and outer one-third of the molecular layer of the dentate gyrus (dg). Some staining was also present in the stratum oriens in CA3 area and in the stratum lacunosum-moleculare the hippocampus proper. Axons in the fimbria (f) were also immunopositive for mGluR4a. As shown in B, immunoreactivity for mGluR4a is totally absent in mouse lacking mGluR4. In **C** is shown the staining for mGluR4a in the cerebellum. Very intense immunoreactivity product was present in the molecular layer (ml), whereas Purkinje cells (Pj) and granular layer (gl) were depleted of mGluR4a immunostaining. In mutant mouse cerebellum there was no immunoreactivity product (**D**). Scale bars = 0.4 mm in A, B; 0.1 mm in C, D.

these regions, mGluR4a immunoreactivity was highly localized on long processes, along which there was a intense staining of punta (Fig. 6). A moderate level of immunoreactivity was present in the STR.

Fig. 4. Immunocytochemical analysis of metabotropic glutamate receptor (mGluR4a) in mouse brain. **A** shows immunocytochemical analysis of staining with antibodies in sagittal sections of wild type mouse brain. This receptor was specifically present in only a few brain regions. The highest level of immunoreactivity was found in the molecular layer of the cerebellum. Intense staining was detected in fibers and puncta in the globus pallidus (GP) and substantia nigra pars reticulata (SNpr) and entopeduncular nucleus (ET). Some immunoreactivity product was also present in the hippocampus. Additionally, mGluR4a immunoreactivity was lower in regions such as the neocortex, thalamus, and striatum (STR). **B** shows that there was no staining in mouse lacking mGluR4. **C** shows that the staining was completely abolished when the mGluR4a antibodies were preadsorbed with 10 μg/ml of homologous peptide. Pir, piriform cortex; Pr5VL, principal sensory trigeminal nucleus; Sp5, spinal trigeminal tract; VLL, ventral nucleus of the lateral lemniscus. Scale bar = 0.5 mm.

Limbic cortical regions also showed relatively strong mGluR4a immunoreactivity. In the hippocampus (Figs. 4A, 5A), mGluR4a reaction product was visible in the outer and middle thirds of the molecular layer in the dentate gyrus. Some staining was also present in the neuropil of the stratum lacunosum-moleculare in area CA1 of the hippocampus proper and the stratum oriens in CA3 area. In the stratum pyramidale, mGluR4a immunoreactivity was noted in puncta surrounding the cell bodies of neurons and less frequently on cell bodies. This type of staining is also present in the granule cells of the dentate gyrus. Finally, mGluR4a immunoreactivity is present in the fimbria. The hippocampus of mice lacking mGluR4 was completly depleted of mGluR4a immunoreactivity (Fig. 5B). A moderate level of immunoreactivity was present in the piriform cortex (Pir), where immunoreactive fibers were present in layer 1A (Fig. 4A).

The highest level of mGluR4a immunoreactivity was found in the molecular layer of the cerebellum (Figs. 4A, 5C). In this region, intense staining was detected in fibers

and neuropil of the molecular layer. In contrast, the cells of the granule layer were virtually devoid of mGluR4a immunoreactivity, as were the cells bodies and dendrites of the Purkinje cells. In the same area, mGluR4a immunoreactivity was absent in mice lacking mGluR4 (Figs. 4B, 5D). Moderate staining was also present in the ventrolateral part of the principal sensory trigeminal nucleus (Pr5VL; Fig. 4A).

Weak mGluR4a immunoreactivity was also present in the spinal trigeminal tract (Sp5) and in the ventral nucleus of the lateral lemniscus (VLL). mGluR4a immunoreactivity was low in several brain regions, including neocortex and thalamus (Fig. 4A).

mGluR4a immunoreactivity in the BG

To further address the pre- vs. postsynaptic distribution of mGluR4a immunoreactivity in rat BG, more detailed fluorescence and electron microscopic studies were performed. Confocal fluorescence microscopy showed intense mGluR4a immunoreactivity in fibers in the GP (Fig. 6). The STR overall exhibited very low intensity staining. Occasional large labeled fibers were observed traversing the border of the STR with the GP. Only very low intensity staining was seen in the SNpr, and the large neurons of the SNpc were not detectably labeled. No mGluR4a immunoreactivity associated with the somata was observed in any of these BG nuclei.

Double label experiments with MAP2 (Huber and Matus, 1984), a dendritic marker (Fig. 7A, B: mGluR4a staining in red in each of the panels of Fig. 7) showed a selective concentration of mGluR4a staining along the margin of certain dendrites in GP. At higher magnification (Fig. 7B), it was clear that most of the mGluR4a immunoreactivity was found on the dendritic surface, as it did not colocalize with MAP2 present within the dendrites. Figure 7C shows double label staining for mGluR4a and Enk. This study demonstrates that only a small subset of the Enk terminals label for mGluR4a. Figure 7D is a high magnification illustration showing double labeling with mGluR4a antibodies and the presynaptic marker SV2. mGluR4a and SV2 immunoreactivity are clearly colocalized along the outside of dendrites in the GP, suggesting a presynaptic localization of mGluR4a in GP. Consistent with this, Figures 7E and 7F show mGluR4a staining in the GP, contralateral (control) and ipsilateral to a quinolinate lesion of the striatum. Quinolinate lesioning of the projecting neurons to GP from the STR induced a marked decrease in mGluR4a immunoreactivity in ipsilateral (Fig. 7F) but not contralateral (Fig. 7E) GP. The residual staining for mGluR4a in the GP after striatal lesioning differed in appearance from the unlesioned side and that found in normal animals. It no longer outlined dendrites, but was instead found in coarse granules within the neuropil of the GP (Fig. 7E). These may represent staining associated with degenerating nerve terminals.

These double-label and lesion studies suggest that mGluR4a may be presynaptically localized on striatopallidal terminals. To further test this hypothesis, we performed immunocytochemistry with analysis at the electron microscopy level to clarify the pre- vs. postsynaptic localization of mGluR4a in the GP. Electron microscopy (EM) revealed that mGluR4a immunoreactivity is presynaptically localized (Fig. 8). Analysis of mGluR4a immunoreactivity at the EM level shows that the most intense staining was on axon terminals that form symmetric synapses with dendrites (Fig. 8A– E). Although the majority of dendrites were not labeled, occasionally light staining of dendritic elements was observed (not shown).

DISCUSSION

In the present studies, we have characterized antibodies that are highly specific for mGluR4a and used them for immunocytochemical analysis of mGluR4a immunoreactivity in mouse and rat brain. Several experiments suggest that the antibodies react in a highly specific manner with the targeted receptor. Western blot analysis revealed that the antibodies selectively recognize proteins in cell lines transfected with mGluR4a but not in nontransfected cells or cell lines transfected with other mGluR subtypes. The antibodies also recognize a native protein in mouse and rat brain homogenates with a molecular weight consistent with the molecular weight of the immunoreactive protein in transfected cell lines. Furthermore, preadsorption of the antibodies with the homologous peptide abolished all the immunoreactive bands and immunocytochemical staining, suggesting that the antibodies react with the targeted epitope rather than binding nonspecifically. Finally, there was no immunoreactivity detected in brains of mutant mice lacking mGluR4. Taken together, these data suggest that these antibodies are highly specific for mGluR4a relative to other mGluR subtypes or other brain proteins.

In this paper, we focus on the localization of mGluR4a in BG because our immunocytochemical data and lesion studies reveal that mGluR4a has an important function as a presynaptic modulator in striatopallidal projections in the BG. The immunocytochemical analysis shows a striking immunoreactivity for mGluR4a in the GP, whereas SNpr and ET, the major output nuclei of the BG, were only weakly immunoreactive to mGluR4a. Finally, there was very low mGluR4a staining in STR or other BG structures. GP, SNpr, and ET each receive inhibitory GABAergic projections from the striatum (Fig. 9). Thus, the present data, coupled with previous in situ hybridization studies (Testa et al., 1994), suggest that mGluR4a could be synthesized in striatal neurons and targeted to presynaptic terminals of striatal projections to these other BG structures. Dual-label in situ studies on mGluR4 mRNA in striatal neurons reveal that the mRNA is present in all striatal projection neurons (Kerner et al., 1997). However, there is little mGluR4a immunoreactivity in the STR. Furthermore, mGluR4a seems to be found only on some of the striatopallidal terminals. These two observations suggest that either only some striatal neurons synthesize the protein, or all of them synthesize mGluR4a, but target it to only selected synapses with a subset of pallidal dendrites. Consistent with this latter hypothesis, in double labeling experiments with MAP2, mGluR4a staining was found clustered along a subset of the dendritic structures. All of the fibers displaying mGluR4a staining also exhibited colocalized staining for Enk, but there were many Enk-positive pallidal fibers and terminals which did not stain for mGluR4a. Thus, it is likely that mGluR4a is present in only a subpopulation of striatopallidal terminals. Double labeling immunocytochemistry revealed colocalization of mGluR4a immunoreactivity with the presynaptic marker synaptophysin. Furthermore, quinolinic acid lesions of the striatum virtually abolished mGluR4a immunoreactivity in GP. Finally, immuno-EM studies revealed that mGluR4a in GP is predominantly localized in presynaptic terminals

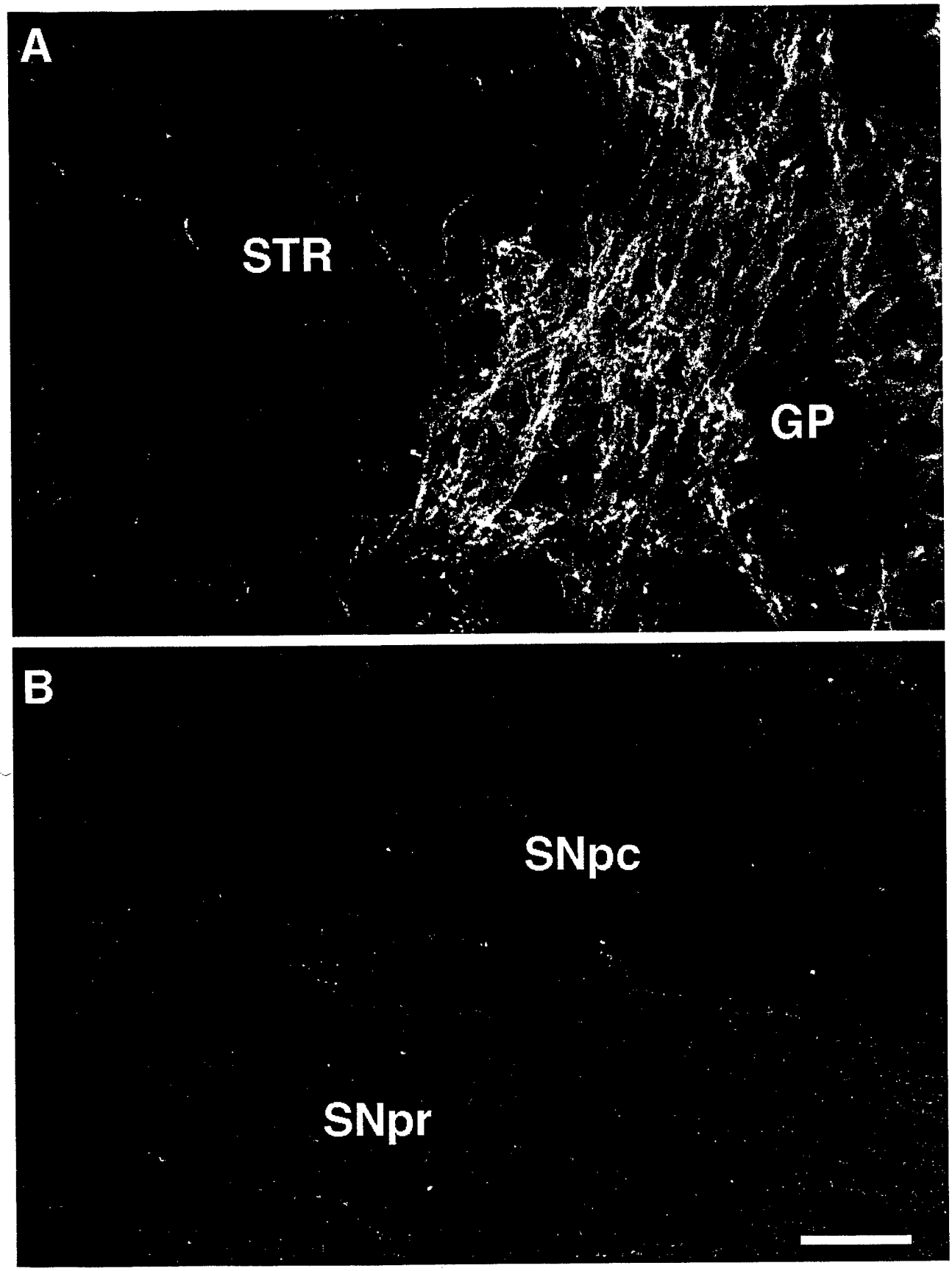

Fig. 6. mGluR4a immunoreactivity in basal ganglia. **A** shows intense localization of metabotropic glutamate receptor (mGluR4a) in fibers in the rat globus pallidus (GP). In contrast, the striatum (STR) is practically devoid of mGluR4a immunoreactivity. **B** shows a light mGluR4a immunoreactivity in the substantia nigra part reticulata (SNpr), and a very light immunoreactivity in the substantia nigra pars compacta (SNpc). Scale bar = 50 mm.

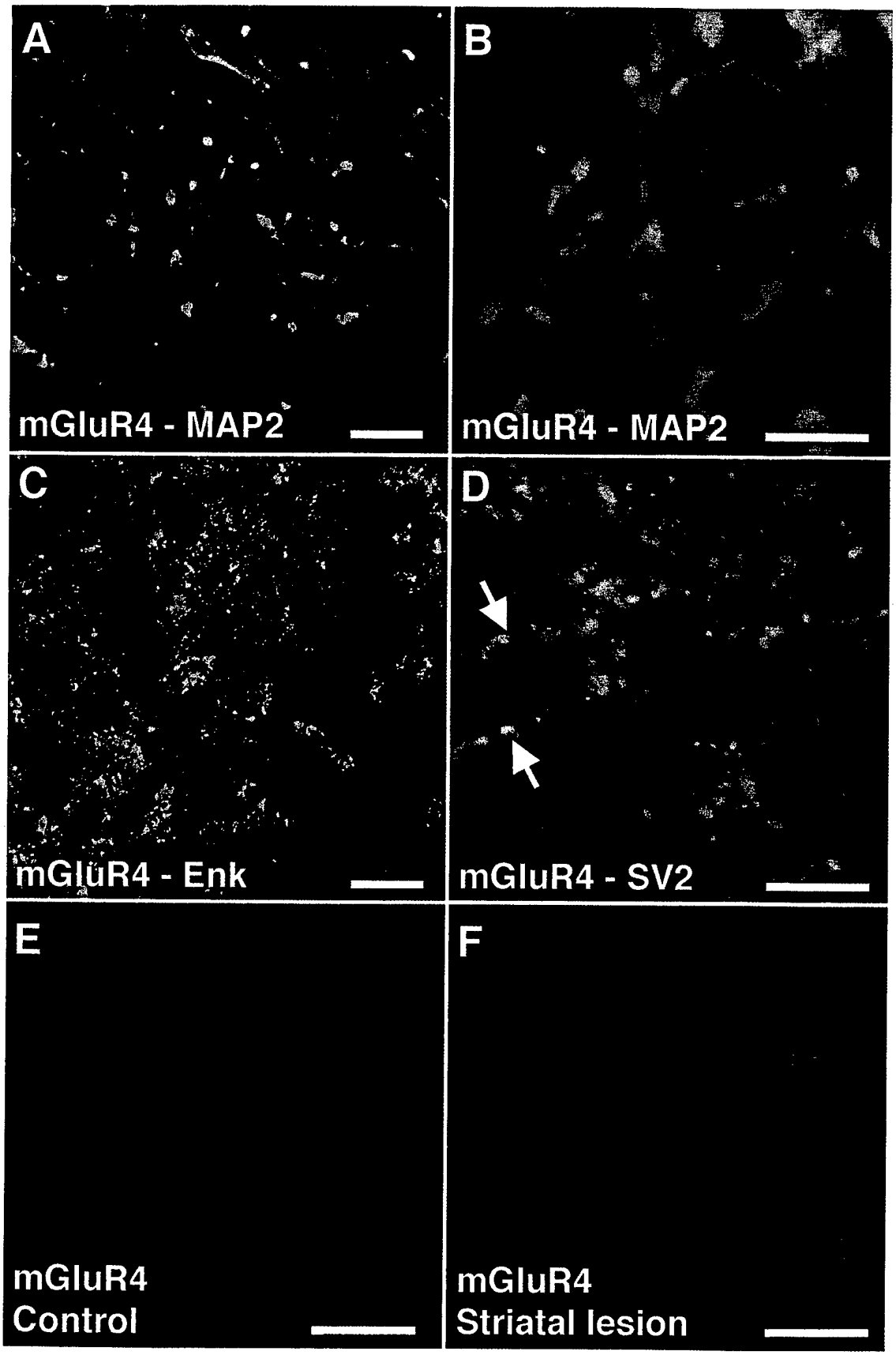

Fig. 7. Double and single labeling confocal microscopy in globus pallidus (GP). **A** shows the selective concentration of metabotropic glutamate receptor (mGluR4a) staining (red) along the outside of certain dendrites (green), labeled with the postsynaptic marker microtubule-associated protein 2 (MAP2). **B** shows at higher magnification the colocalization of mGluR4a and MAP2. **C** shows staining for enkephalin (Enk; green), demonstrating that only a few Enk terminal terminals are labeled for mGluR4a (red). **D** is a high magnification photo showing the colocalization (arrows) outside a dendrite of the presynaptic marker synaptic vesicle protein 2 (SV2; green) and mGluR4a (red), suggesting the presynaptic localization of mGluR4a in GP. **E** and **F** are illustrations of mGluR4a staining (red) in GP, contralateral (E) and ipsilateral (F) to a quinolinate lesion of the striatum. Scale bars = 10 mm in A, C; 5 mm in B, D; 100 mm in E, F.

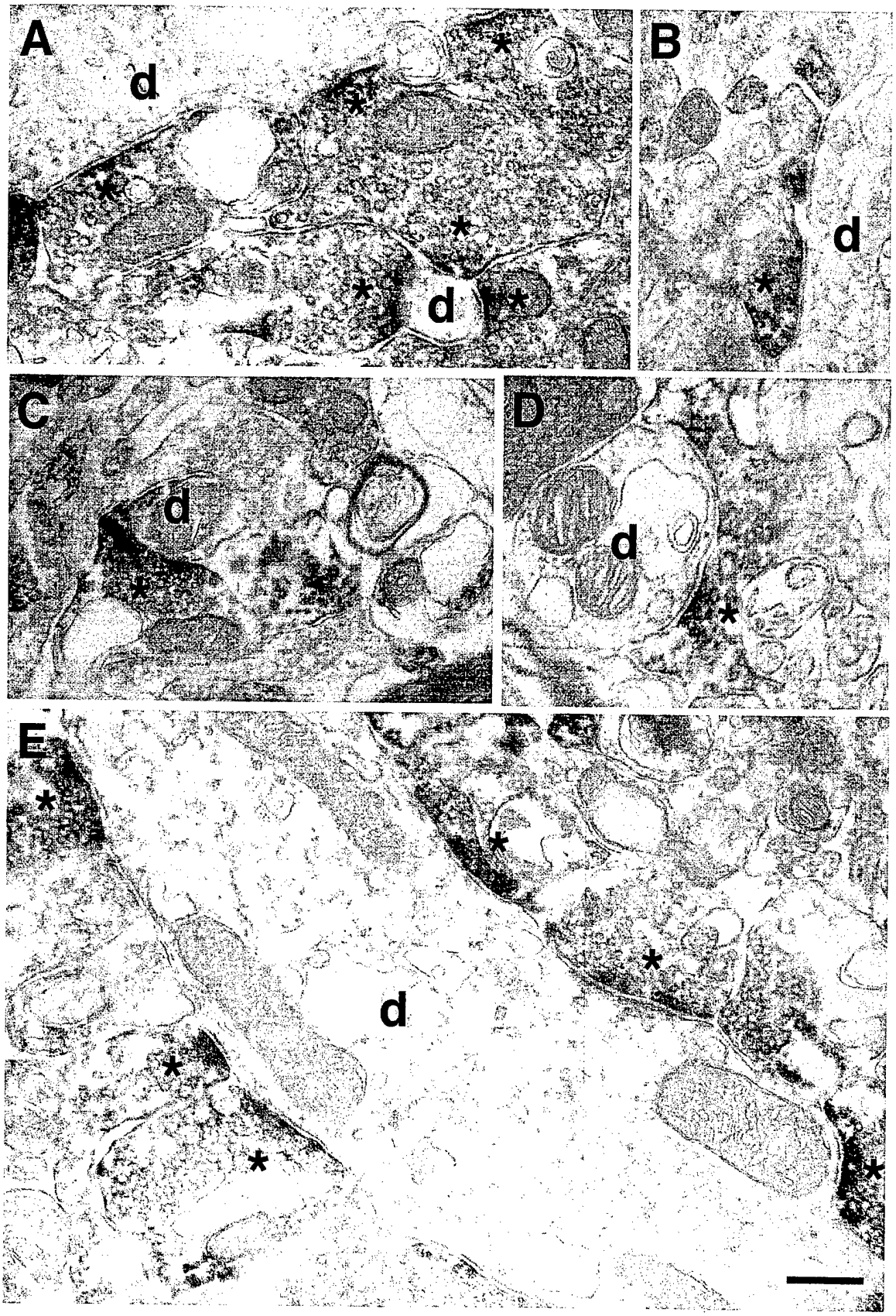

Fig. 8. **A–E:** Electron micrographs demonstrating presynaptic metabotropic glutamate receptor (mGluR4a) immunoreactivity in the globus pallidus (GP). Examples of mGluR4a axon terminals (asterisks) synapsing with dendrites (d) of cells in the GP. Scale bar = 400 nm in A–D; 350 nm in E.

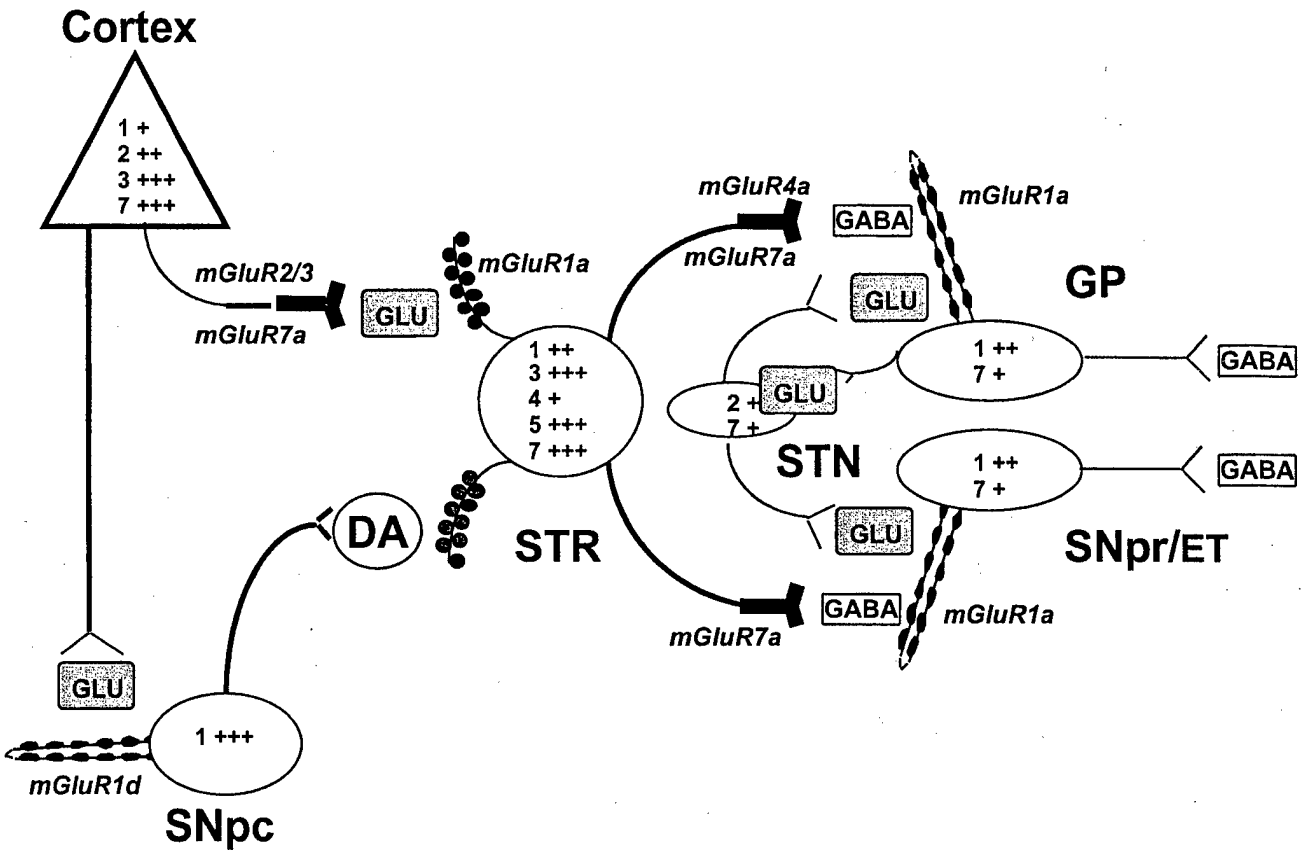

Fig. 9. Summary figure illustrating results of the present and previous studies of metabotropic glutamate receptor mGluR localization in basal ganglia. Illustrated in schematic form are the two principal sources of afferent projections to striatum (STR), the cerebral cortex, which employs glutamate (GLU) as a transmitter, and the dopaminergic (DA) projection from the substantia nigra pars compacta (SNpc). The striatal projection neurons employ g-amino-n-butyric acid (GABA) as a neurotransmitter, and project to the output nuclei entopeduncular nucleus/substantia nigra part reticulata (ET/SNpr/ET) either directly, or through a disynaptic relay involving the globus pallidus (GP) and the subthalamic nucleus (STN). Subthalamic efferents provide excitatory glutamatergic input to the GP and SNpr. In this schematic, the labels 1 to 7 refer to mRNAs encoding the mGluRs, whereas the symbols (+, ++, and +++) indicate the relative intensity of expression as determined by hybridization. Immunoreactivity is represented as *mGluR–mGluR7*. mGluR1 (most likely the mGluR1d isoform) is found on the dendrites of dopamine neurons in the SNpc, but appears not to be present on the axonal processes on these cells. Intense mGluR4a and mGluR7a immunoreactivity was detected in association with striatal terminals in GP.

that resemble striatal boutons and form symmetric synapses with GP dendrites and spines. Taken together, these data provide strong evidence that mGluR4a is localized on striato-pallidal terminals synapsing onto a subset of pallidal dendrites.

A comparison between these results and recent studies from our laboratories of mGluR7a localization in BG (Kosinski et al., 1999) reveals a number of similarities between the distribution of mGluR4a and mGluR7a. For instance, double label in situ hybridization studies revealed that mGluR7 mRNA is abundantly expressed in striatal projection neurons but not in striatal interneurons. As in the present experiments with mGluR4a, double labeling immunocytochemistry revealed colocalization of mGluR7a immunoreactivity with the presynaptic marker synaptophysin, and quinolinic acid lesions of the striatum resulted in the loss of mGluR7a immunoreactivity in GP. Finally, immuno-EM studies revealed that mGluR7a immunoreactivity is present in presynaptic terminals at symmetric synapses in GP. However, there are also several important differences between mGluR4a and mGluR7a immunoreactivity. For instance, mGluR7a immunoreactivity is intense in STR, and lesion studies suggest that this represents staining at corticostriatal synapses. In contrast, there is little mGluR4a immunoreactivity in STR. Also, mGluR4a immunoreactivity is abundant in GP (likely at striatopallidal synapses) but relatively weak in substantia nigra (SN). In contrast, mGluR7a immunoreactivity is equally strong in SN and GP. Also, EM analysis revealed both pre- and postsynaptic labeling for mGluR7a in both GP and STR, whereas mGluR4a immunoreactivity was almost exclusively presynaptic.

Taken together, the present studies and the previous studies suggest that both mGluR4a and mGluR7a are localized in presynaptic terminals of striatopallidal projections. Previous studies show that a common function of mGluRs observed in other brain regions is a role as presynaptic heteroreceptors on inhibitory nerve terminals involved in inhibition of GABA release (Desai and Conn, 1991; Calabresi et al., 1992; Desai et al., 1994; Stefani et al., 1994). Thus, it is possible that presynaptically localized mGluR4a and mGluR7a could serve as heteroreceptors involved in regulating GABA release from striatopallidal and striato-SNpr terminals. The possibility that

different mGluR subtypes may serve as heteroreceptors at different synapses is particularly intriguing and raises the possibility of selectively targeting the mGluR subtypes that serve as heteroreceptors in specific neuronal circuits relevant for various disorders. For instance, as a potential treatment for PD, agonists of group III receptors would reduce GABAergic inhibition of GP neurons, leading to an increase in firing of GABAergic GP neurons that project to the STN. This increased GABAergic inhibition of STN neurons would reduce STN neuronal firing and could normalize the over activity of STN neurons that occurs in PD. If so, these mGluRs could provide novel targets for new therapeutic agents that could be useful in treatment of PD and other disorders of BG function. However, the therapeutic benefit of group III mGluR agonists may ultimately depend on the balance of effects in GP and SNpr. If group III mGluRs also serve as presynaptic heteroreceptors on GABAergic terminals in SNpr, this could counteract the beneficial effect of activation of these receptors in GP.

Although the antibodies used for immunocytochemical analysis in the present study were highly specific for mGluR4a, other antibodies raised against the same epitope showed clear cross-reactivity with a band in homogenates from mutant mice that lacked mGluR4a. Interestingly, this cross-reactive band comigrated with mGluR4a, making it impossible to distinguish from mGluR4a except in the mutant mice. In a previous study (Bradley et al., 1996), we reported a regional and cellular distribution of immunoreactivity with mGluR4a-directed antibodies that appear to cross-react with this band in mGluR4 mutant mice. Western blot analysis with the previously characterized antibodies was relatively uniform in most brain regions and was clearly different from the distribution of mGluR4 mRNA (Tanabe et al., 1993). Immunocytochemical staining with these antibodies was also distinct from that reported here, and mGluR4a appears to have a more restricted distribution to presynaptic sites then we previously reported.

The identity of the mGluR4a cross-reactive protein is unknown. However, there are several interesting properties of the protein that raise the possibility that this protein it might be related to the mGluRs. First, it shares immunological features with mGluR4a. Second, the cross-reaction protein has essentially identical mobility of mGluR4a by SDS-PAGE, indicating that their molecular weights are very similar. Third, the appearance of both bands is typical of glycosylated proteins. Fourth, ultrastructural analysis of the distribution of the cross-reacting protein implies that it is a membrane protein and postsynaptically localized at putative glutamatergic synapses. Taken together, these data raise the possibility that this protein may be related to mGluRs and may serve a postsynaptic function at glutamatergic synapses. Because the antibody does not cross-react with any known mGluRs in transfected cells, it may represent a novel mGluR or mGluR-like protein.

In summary, the present data suggest that mGluR4a has an abundant, but highly localized, distribution in BG. Taken together with previous studies focused on other mGluR subtypes, these data suggest that mGluRs are richly distributed in BG structures where they could play important roles in regulating the net output of these structures. Figure 9 summarizes our current understanding of mGluR localization in BG. Group III mGluRs (mGluR4 and mGluR7) are predominantly localized on presynaptic terminals of striatal projections to GP and SNpr (present studies; Kosinski et al., 1999). Group II mGluRs are presynaptically localized on corticostriatal projections and are expressed in STR (mGluR3) and STN (mGluR2), suggesting that they could play a role in these regions or in terminals of neurons originating from these areas, such as GP and SNpr (Testa et al., 1998). Finally, group I mGluRs are abundantly expressed in many of the BG nuclei (SNpc, SNpr, STR, and GP) where they are likely to serve as postsynaptic receptors involved in regulating neuronal excitability (Testa et al., 1998). If future electrophysiology studies verify the physiological roles of these receptors that would be predicted from these anatomical studies, this will provide a strong basis for development of novel therapeutic agents that target specific mGluR subtypes and could be used for treatment of PD and other disorders involving pathological changes in BG function.

ACKNOWLEDGMENTS

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Activation of Group II Metabotropic Glutamate Receptors Inhibits Synaptic Excitation of The Substantia Nigra Pars Reticulata

Stefania R. Bradley*, Michael J. Marino*, Marion Wittmann†, Susan T. Rouse, Allan I. Levey‡, and P. Jeffrey Conn.

Departments of Pharmacology, and ‡Neurology, Emory University, Atlanta, GA 30322 and † Tierphysiologie, University of Tuebingen, D-72076 Tuebingen, Germany.

*These authors contributed equally to this work.

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Address Correspondences to: Dr. P.J. Conn
Department of Pharmacology
Rollins Research Building
Atlanta, GA 30322
(404) 727-5991
Fax: (404) 727-0365
email: pconn@emory.edu

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ABSTRACT

Loss of nigro-striatal dopaminergic neurons in Parkinson's disease (PD) leads to increased activity of glutamatergic neurons in the subthalamic nucleus (STN) . Recent studies reveal that the resultant increase in STN-induced excitation of basal ganglia output nuclei is responsible for the disabling motor impairment characteristic of PD. Based on this, it is possible that that any manipulation that reduces activity at excitatory STN synapses onto basal ganglia output nuclei could be useful in treatment of PD. We now report that group II metabotropic glutamate receptors (mGluRs) are presynaptically localized on STN terminals and that activation of these receptors inhibits excitatory transmission at STN synapses. Consistent with the hypothesis that this could provide a therapeutic benefit in PD, a selective agonist of a group II mGluRs induces a dramatic reversal of catalepsy in a rat model of PD. These results raise the exciting possibility that selective agonists of group II mGluRs could provide an entirely new approach to the treatment of PD. These novel therapeutic agents would provide a non-invasive pharmacological treatment which does not involve the manipulation of dopaminergic systems, thus avoiding the problems associated with current therapies.

Key Words: Substantia Nigra Pars Reticulata, Subthalamic Nucleus, Group II Metabotropic Glutamate Receptors, Parkinson's Disease, Catalepsy, Presynaptic Inhibition.

Parkinson's disease (PD) is a common neurodegenerative disorder characterized by disabling motor impairments including tremor, rigidity, and bradykinesia. The primary pathological change giving rise to the symptoms of Parkinson's disease is loss of dopaminergic neurons in the substantia nigra pars compacta that modulate the function of neurons in the striatum and other nuclei in the basal ganglia (BG) motor circuit. Currently, the most effective pharmacological agents for treatment of PD include levodopa (L-DOPA), the immediate precursor of dopamine, and other drugs that replace the lost dopaminergic modulation of BG function (Poewe and Granata, 1997). Unfortunately, dopamine replacement therapy ultimately fails in most patients due to loss of efficacy with progression of the disease and severe motor and psychiatric side effects (Poewe et al., 1986). Because of this, a great deal of effort has been focused on developing new approaches for treatment of PD.

Recent studies reveal that loss of nigrostriatal dopamine neurons results in a series of neurophysiological changes that lead to over activity of a critical nucleus in the BG motor circuit termed the subthalamic nucleus (STN). The STN contains glutamatergic projection neurons that provide the major excitatory input to the globus pallidus internal capsule (GPi) and the substantia nigra pars reticulata (SNr), the major output nuclei of the basal ganglia. Increased activity of STN neurons leads to an increase in glutamate release at STN synapses onto GABAergic projection neurons in the output nuclei. This glutamate-mediated over excitation of BG output ultimately leads to a "shutdown" of thalamocortical projections and produces the motor impairment characteristic of PD (Wichmann and DeLong, 1997). Discovery of the pivotal role of increased STN-mediated excitation of the BG output nuclei in PD has led to a major focus on surgical approaches for treatment. For instance, lesions or high frequency stimulation of the STN provides a therapeutic benefit to PD patients (Limousin et al., 1995). In addition, pallidotomy, a surgical

lesion of the GP, produces similar therapeutic effects by reversing the impact of increased activity of STN neurons (Laitinen et al., 1992; Baron et al., 1996). Development of these highly effective neurosurgical approaches provides a major advance in our understanding of the pathophysiology of Parkinson's disease. However, surgical approaches are not widely available to Parkinson's patients. Due to their invasive nature, high cost, and considerable expertise required, such treatment is reserved for patients that are refractory to dopamimetic therapy.

An alternative to surgical approaches to reducing the increased excitation of basal ganglia output nuclei in PD patients would be to employ pharmacological agents that counteract the effects of over activation of the STN neurons by reducing transmission at excitatory STN synapses onto the SNr and GPi neurons. While antagonists of postsynaptic ionotropic glutamate receptors can improve parkinsonian symptoms in PD patients and in animal models of PD (Klockgether et al., 1993; Kornhuber et al., 1994) these compounds are most effective as adjuncts to dopamine replacement therapy (Starr, 1995). Another approach would be to target metabotropic glutamate receptors (mGluRs), which are often localized presynaptically on glutamatergic terminals where they can inhibit glutamate release. Interestingly, the group II mGluRs (mGluR2 and mGluR3) are expressed in STN neurons (Testa et al., 1994) and these receptors have been shown to regulate glutamate release in other brain regions (Hayashi et al., 1993; Shigemoto et al., 1997). We now report that group II mGluRs are presynaptically localized on STN terminals in the SNr and that activation of these receptors reduces excitatory synaptic responses. Furthermore, activation of group II mGluRs provides a dramatic therapeutic effect in a rat model of Parkinson's disease. If this, or related drugs prove to be effective in patients with Parkinson's disease, this could lead to a novel approach for treatment of this debilitating disorder.

MATERIALS AND METHODS

Materials

6-Cyano-7-nitroquinoxaline-2,3-dione (CNQX), (RS)-α-Cyclopropyl-4-phosphonophenylglycine (CPPG), D(-)-2-Amino-5-phosphonopentanoic acid (D-AP5), and (2S,2'R,3'R)-2-(2',3'-Dicarboxycyclopropyl)glycine (DCG-IV) were obtained from Tocris (Ballwin, MO). 2R,4R-4-aminopyrrolidine-2,4-dicarboxylate (APDC), (+)-2-aminobicyclo[3.1.0]-hexane-2,6-dicarboxylate monohydrate (LY354740), and 2S-2-amino-2-(1S, 2S-2-carboxycyclopropyl-1-yl)-3-(xanth-9-yl)propanoic acid (LY341495) were gifts from D. Schoepp and J. Monn (Eli Lilly, Indianapolis, IN). All other materials were obtained from Sigma (St. Louis, MO).

Electrophysiology

Whole-patch clamp recordings were obtained as previously described (Marino et al., 1998) except that recordings were made under visual control. 15-18 day old Sprague-Dawley rats were used for all patch clamp studies. Brains were rapidly removed and submerged in an ice cold sucrose buffer (in mM: Sucrose, 187; KCL, 3; $MgSO_4$, 1.9; KH_2PO_4, 1.2; Glucose, 20; $NaHCO_3$, 26; equilibrated with 95% O_2/5% CO_2). Parasaggital slices (300μM thick) were made using a Vibraslicer (WPI). Slices were transferred to a holding chamber containing normal ACSF (in mM: NaCl, 124; KCL, 2.5; $MgSO_4$, 1.3; NaH_2PO_4,1.0; $CaCl_2$, 2.0; Glucose, 20; $NaHCO_3$, 26; equilibrated with 95% O_2/5% CO_2). In some experiments, 5 μM glutathione, 500 μM pyruvate, and 250 μM kynurenate were included in the sucrose buffer and holding chamber. These additional compounds tended to increase slice viability but did not have any effect on

experimental outcome. Therefore all of the data from these two groups have been pooled. Slices were transferred to the stage of a Hoffman modulation contrast microscope and continually perfused with room temperature ACSF (~3 mL/min, 23-24°C). Neurons in the substantia nigra pars reticulata were visualized with a 40X water immersion lens. Patch electrodes were pulled from borosilicate glass on a Narashige vertical patch pipette puller and filled with (in mM: potassium gluconate, 140; HEPES, 10: NaCl, 10; EGTA, 0.6; GTP, 0.2; ATP, 2; pH adjusted to 7.5 with 0.5 N NaOH). Biocytin (0.5%, free base) was added just prior to use. Electrode resistance was 3-7 MΩ. For measurement of synaptically evoked currents, bipolar tungsten electrodes were used to apply stimuli to the STN. Stimulating electrodes were positioned with one pole slightly penetrating the tissue, and the other pole above the slice. Synaptically evoked excitatory postsynaptic potentials (EPSCs) were record from a holding potential of -60 mV and slices were bathed in 50 μM picrotoxin. Inhibitory post synapstic potentials (IPSCs) were evoked in a similar manner but with the electrodes placed in the cerebral peduncle rostral to the recording sight, and in the presence of 10 μM CNQX and 10 μM D-AP5 to block excitatory transmission. IPSCs were recorded from a holding potential of -50 mV. For measurement of kainate-evoked currents kainate (100 μM) was pressure ejected into the slice from a low resistance pipette. Kainate-evoked currents were recorded from a holding potential of -60 mV, and slices were bathed in ACSF containing 500nM tetrodotoxin. For studies of mEPSCs, slices were bathed in standard ACSF with the addition of 50 mM mannitol, 500 nM tetrodotoxin, and 10 μM bicuculline warmed to 25° C. GABAergic projection neurons were identified according to previously established electrophysiological and morphological criteria (Richards et al., 1997). GABAergic neurons exhibited spontaneous repetitive firings, short duration action potentials (half amplitude duration = 1.7 ±0.2 msec), little spike frequency adaptation, and a lack of inward

rectification, while dopaminergic neurons displayed no, or low frequency spontaneous firings, longer duration action potentials (half amplitude duration = 7.0 ± 0.5 msec), strong spike frequency adaptation, and a pronounced inward rectification. Light microscopic examination of biocytin filled neurons indicated that GABAergic neurons had extensive dendritic arborizations close to the cell body, whereas the dendritic structures of dopaminergic neurons were relatively sparse. All of the data presented in these studies are from confirmed GABAergic neurons.

Immunocytochemistry

Preparation of the tissue for immunocytochemistry analysis at electron microscopy level was obtained following previously published protocols (Bradley et al., 1996). The avidin-biotin-peroxidase method (Vectastain Elite ABC kit, Vector laboratories, Burlingame, CA) was used to detect mGluR2/3 immunoreactivity in rat (n=2) SNr. The peroxidase reaction was developed in 0.05% diaminobenzidine and 0.01% H_2O_2. Antibodies that specifically recognize mGluR2 and mGluR3 are from Chemicon (Temecula, CA).

Behavioral studies

Male Sprague Dawley rats 30 days old at the start of experiments were injected i.p. with either haloperidol (2 mg/ml solution dissolved in 8.5% lactic acid, neutralized with 1 N NaOH, and diluted to 0.3 mg/ml in saline) or saline, and returned to their home cage for 30 minutes. After 30 minutes, the animals were injected with either saline, or LY354740 (0.6-6 mg/ml dissolved in saline). Catalepsy was measured 1 hour later by placing the animals forepaws on a bar elevated 4.5 cm. The time to removal of one paw was measured by a stopwatch. Animals were then placed on a vertical mesh approximately 6 inches above ground and the time to remove

one paw from the mesh was measured. Animals were tested once per day and saline controls were run between each drug test. All animals were habituated to the tasks by three consecutive days of saline control treatment before beginning drug testing.

RESULTS

Whole cell patch clamp techniques were used to record excitatory postsynaptic currents (EPSCs) from GABAergic projection neurons of the SNr in midbrain slices. EPSCs were elicited by stimulation of the STN with bipolar stimulating electrodes (0.4 - 12.0 μA every 60-90 seconds) in the presence of 50 μM picrotoxin. EPSCs elicited with this protocol had a constant latency, were monophasic, and were completely abolished with application of 10 μM CNQX (n=10, data not shown), suggesting that the synaptic response was a monosynaptic glutamatergic EPSC.

Activation of group II mGluRs inhibits transmission at the STN-SNr synapse.

Application of 100 nM LY354740, a highly selective agonist of group II mGluRs (Monn et al., 1997), produced a reversible depression of EPSCs in SNr projection neurons (Figure 1). A concentration response curve for LY354740 revealed an EC_{50} of approximately 75 nM (Figure 1B), consistent with the potency of this compound at group II mGluRs. The reduction of EPSC amplitude was mimicked by two other highly selective agonists of group II mGluRs, 2R,4R-APDC (Schoepp et al., 1995), and DCG-IV (Hayashi et al., 1993; Gereau and Conn, 1995) (Figure 1C), and was completely blocked by prior application of LY341495 (100 nM) or CPPG; (500 μM) (Figure 2) both of which are antagonists active at group II mGluRs (Kingston et al., 1998; Toms et al., 1996).

Group II mGluRs are localized presynaptically at excitatory terminals in the SNr.

Taken together, these data suggest that activation of group II mGluRs reduces transmission at the STN-SNr synapse. We used a combination of immunocytochemical and biophysical approaches to determine whether group II mGluRs elicit this effect by a presynaptic or a postsynaptic

mechanism of action. First, we employed antibodies that specifically recognize both mGluR2 and mGluR3 for immunocytochemical localization of group II mGluRs in the SNr. Analysis of mGluR2/3 immunoreactivity at the electron microscopic (EM) level revealed that group II mGluRs are presynaptically localized (Figure 3). The morphology of the labeled synapses, including their assymetric nature, was characteristic of STN terminals (Fig 3A-D)(Bevan et al., 1994). We also observed labeling of terminals that did not make clear synaptic contact with postsynaptic elements and of fine processes that were reminiscent of previous reports of mGluR2/3 distribution in preterminal axons (data not shown)(Lujan et al., 1997). Furthermore, there was occasional labeling of symmetric synapses (Fig 3E), though the majority of symmetric synapses were unlabeled. There was no observable staining of dendrites, dendritic spines, or other postsynaptic elements.

The group II mGluR-mediated inhibition of synaptic transmission is due to a presynaptic mechanism. The presence of mGluR2/3 immunoreactivity at presynaptic but not postsynaptic sites in SNr suggests that these receptors are likely to act by inhibiting glutamate release from presynaptic terminals rather than by modulating the postsynaptic glutamate-gated cation channels. To further test this hypothesis, we determined the effects of maximal concentrations of LY354740 on currents elicited by brief (50-500 msec) pressure ejection of kainate (100 μM) into the slice. In the presence of 500 nM tetrodotoxin, kainate application produced a robust, stable, inward current in SNr GABAergic neurons (Figure 4A). Application of 100 nM LY354740 produced no significant change in kainate-evoked currents (Figure 4A, B).

While the lack of effect of LY354740 on kainate-evoked currents is consistent with a presynaptic mechanism of action, it is conceivable that exogenously applied kainate selectively

activates non synaptic glutamate receptor channels and that LY354740 selectively modulates channels that are localized at synapses. Thus, we also determined the effect of maximal concentrations of LY354740 on frequency and amplitude of spontaneous miniature EPSCs (mEPSCs). Recordings were made in the presence of tetrodotoxin (500 nM) to block activity-dependent release, and bicuculline (10 μM) to block $GABA_A$-mediated synaptic currents. LY354740 (100nM) produced no significant alteration in mEPSC frequency, amplitude, or waveform (Figure 5A-C) suggesting that group II mGluR agonists act presynaptically to reduce transmission. This can be observed by a lack of effect of LY354740 on either the amplitude histograms (Figure 5C) or the cumulative probability plots (Figure 5D). Furthermore, overlay of an average of all mEPSCs before and after LY354740 application shows identical current amplitudes and kinetics between the two conditions (Figure 5B). The average mEPSC frequency is 4.71 ± 0.79 Hz before drug application and 4.66 ± 0.8 Hz during application of 100 nM LY354740 ($p>0.05$; n=5). The average amplitude of mEPSCs was 9.2 ± 1.3 pA before and 8.4 ± 0.8 pA after LY354740 addition ($p>0.05$; n=5).

Activation of group II mGluRs has no effect on inhibitory synaptic transmission in the SNr. If group II mGluRs selectively regulate transmission at STN synapses without altering transmission at inhibitory synapses in the SNr, agonists of these receptors would have a net inhibitory effect on excitatory drive through this portion of the basal ganglia circuit. The immunocytochemical data presented above suggest that mGluR2/3 immunoreactivity is not present on the majority of inhibitory synapses in the SNr, suggesting that group II mGluRs are not likely to modulate IPSCs in this region. To directly test this hypothesis, we determined the effect of LY354740 on evoked IPSCs recorded in SNr projection neurons. Consistent with previous

reports (Radnikow and Misgeld, 1998)stimulation of the cerebral peduncle produced a robust, bicuculline-sensitive IPSC (Figure 6A). Application of a concentration of the group II mGluR agonist LY354740 that is maximally effective in reducing EPSCs had no effect on IPSC amplitude. These results suggest that agonists of group II mGluRs will selectively inhibit excitatory transmission through the indirect pathway to the SNr without impacting direct GABA-mediated inhibition of SNr neurons.

Activation of Group II mGluRs exhibits antiparkinsonian effects. The preceding data clearly demonstrate that group II mGluRs mediate a presynaptic inhibition of transmission at the STN-SNr synapse. Since over activity at this synapse is thought to contribute to the motor dysfunction associated with PD and other hypokinetic disorders, we tested the hypothesis that activation of group II mGluRs would increase mobility in a rat model of parkinsonism using haloperidol-induced catalepsy (Ossowska et al., 1990; Schmidt et al., 1997). Two standard behavioral measures were used to assess catalepsy in rats treated with the dopamine receptor antagonist haloperidol. First, the front paws of control and experimental rats were placed on a horizontal bar (4.5 cm high) and the latency to remove a paw from the bar was measured. Second, rats were placed on a vertical grid and the latency to remove a paw from the grid was measured (Kronthaler and Schmidt, 1996). Consistent with previous reports (Ossowska et al., 1990; Schmidt et al., 1997), haloperidol (1.5 mg/kg) induced a robust catalepsy that could be observed as an increase in latency with both behavioral measures (Figure 7). Interestingly, haloperidol-induced catalepsy was reversed in a dose dependent manner by intraperitoneal (IP) injection of the group II mGluR agonist LY354740. Injection of LY354740 alone had no significant effect on these behavioral measures.

DISCUSSION

We have found that group II mGluRs are presynaptically localized on STN terminals in BG output nuclei where they reduce transmission at STN synapses. Furthermore, a selective agonist of group II mGluRs has behavioral effects in rats that are consistent with an antiparkinsonian action. These data suggest that activation of group II mGluRs restores the normal function of BG circuits by acting at a point downstream of the striatum where dopamine receptor blockade occurs.

The finding that LY354740 alone had no effects on measures of catalepsy is interesting in light of previous studies demonstrating that non selective mGluR agonists can induce catalepsy (Kronthaler and Schmidt, 1996). Since LY354740 is highly selective for group II mGluRs, it is likely that this mGluR-induced catalepsy is due to activation of another mGluR subtype. Consistent with this, LY354740 produces no effect on motor activity when administered alone (Helton et al., 1998) but reduces haloperidol-induced muscle rigidity (Konieczny et al., 1998). Furthermore, agonists of group I mGluRs have physiological and behavioral effects that suggest that agonists of these receptors are likely to have catalepsy-inducing effects (Sacaan et al., 1991; Kaatz and Albin, 1995).

Other potential sites of action of group II mGluR agonists. Taken together with previous studies revealing a critical role of the STN in parkinsonian states (Guridi and Obeso, 1997; Wichmann and DeLong, 1998), the results of the present anatomical and physiological studies suggest that the behavioral effects of LY354740 are at least partially due to an mGluR2/3-mediated reduction in glutamate release from STN terminals. However, it is possible that actions of group II mGluR at other sites could also contribute to this effect. While the distribution of

group II mGluRs in other basal ganglia structures is somewhat limited, previous studies reveal that these receptors are present in striatum (Testa et al., 1998) where they are involved in regulating transmission at cortico-striatal synapses (Lovinger and McCool, 1995) (Pisani et al., 1997a). If group II mGluRs are preferentially involved in inhibiting synaptic excitation of striatal projection neurons that give rise to the indirect pathway, this could contribute to the overall behavioral effects of group II mGluR agonists. Also, it is possible that group II mGluRs present in motor regions outside of the basal ganglia, such as the cortex (Neki et al., 1996) and thalamus (Ohishi et al., 1993) could contribute to the motor effects of group II mGluR agonists.

It is interesting to note that, in addition to projecting to basal ganglia output nuclei, STN neurons also project to the dopaminergic neurons of the SNc (Kita and Kitai, 1987; Iribe et al., 1999). Furthermore, glutamate has been implicated as an excitotoxic agent in PD (Albin and Greenamyre, 1992; Rodriguez et al., 1998), suggesting that increased excitatory drive to the SNc may contribute to the progressive loss of SNc dopaminergic neurons in PD. Based on this, if group II mGluRs are also involved in inhibiting transmission at STN synapses in the SNc, it is possible that agonists of these receptors could reduce the component of SNc neuronal death that is mediated by STN-induced excitotoxicity. Interestingly, previous immunocytochemical studies reveal that mGluR2/3 immunoreactivity is present in the SNc (Testa et al., 1998) Furthermore, physiological studies reveal that agonists of group II mGluRs inhibit evoked EPSPs in this region (Wigmore and Lacey, 1998). While the source of the excitatory afferents regulated by group II mGluRs in SNc was not defined, it is possible that these EPSCs are mediated in part by activity at STN terminals. These data raise the exciting possibility that group II mGluR agonists have potential not only for reducing the symptoms of established PD, but could also slow progression of PD. Future studies will be needed to clearly define the role of increased STN activity in

contributing to progression of the disorder and to rigorously define the mGluR subtypes involved in regulating transmission at STN-SNc synapses.

Basal ganglia modulation by other mGluR subtypes. In addition to group II mGluRs, a growing body of evidence indicates that multiple mGluR subtypes are abundantly expressed throughout the BG (Testa et al., 1994; Testa et al., 1995; Kerner et al., 1997; Kosinski et al., 1998; Bradley et al., 1999a; Bradley et al., 1999b) where they play important neuromodulatory roles. Both group I mGluR subtypes are present in various BG nuclei. For instance, mGluR5 is heavily expressed in striatum, and is also present in subthalamic nucleus, and at lower levels in the pallidal complex, and substantia nigra (Testa et al., 1994; Testa et al., 1995; Kerner et al., 1997) (Tallaksen-Greene et al., 1998). While the levels of mGluR1 are more limited, this receptor is also expressed throughout the basal ganglia (Testa et al., 1994; Kerner et al., 1997; Tallaksen-Greene et al., 1998). A number of studies suggest that agonists of group I mGluRs may act at several levels to increase the net activity of projection neurons in the BG output nuclei. For instance, activation of group I mGluRs potentiates NMDA receptor currents in striatal neurons (Colwell and Levine, 1994; Pisani et al., 1997b) and behavioral studies combined with studies of changes in 2-deoxyglucose uptake and fos immunoreactivity suggest that injection of group I mGluR agonists in the striatum induces a selective activation of the indirect pathway from the striatum to the output nuclei (Kaatz and Albin, 1995; Kearney et al., 1997) . Furthermore, group I mGluRs are present in both STN and SNr neurons and recent physiological studies suggest that activation of these receptors has profound excitatory effects in projection neurons in both of these regions (Abbott et al., 1997; Awad and Conn, 1999; Marino et al., 1999). Thus, group I mGluRs may act at least three levels to increase net excitation of neurons in the BG output nuclei.

Less is known about the physiological roles of group III mGluRs in BG nuclei. However, previous studies reveal that mGluR4 and mGluR7 immunoreactivity are abundant in the striatum (Kosinski et al., 1999; Bradley et al., 1999a; Bradley et al., 1999b)and that agonists of group III mGluRs inhibit glutamate release from cortico-striatal terminals (East et al., 1995; Pisani et al., 1997a). Furthermore, both mGluR4a and mGluR7a immunoreactivity are present on terminals of striatal neurons projecting to the GP and to the SNr. Interestingly, mGluR4a immunoreactivity is enriched in striato-pallidal relative to stiato-nigral terminals (Bradley et al., 1999a). If these receptors are involved in reducing GABA release from striatal terminals, mGluR4 could selectively regulate transmission through the indirect pathway.

In summary, the data presented suggest that group II mGluRs are presynaptically localized on STN terminals in the SNr and that activation of these receptors selectively reduces transmission at excitatory STN synapses in this region. Taken together with the behavioral data presented, these studies raise the exciting possibility that agonists of group II mGluRs may provide a novel, non-surgical approach to treatment of PD which bypasses the problems inherent with dopamine-replacement therapy. Furthermore, because group II mGluR agonists act downstream from nigro-striatal dopaminergic neurons, these compounds could be useful for treatment of drug-induced parkinsonism in patients treated with haloperidol and other dopamine receptor antagonists that are used as antipsychotic agents. Finally, it is important to note that pallidotomy and inactivation of the STN are being explored as having therapeutic potential in other movement disorders, including dystonia and tardive dyskinesias (Vitek et al., 1998), and that increased activity in STN is implicated in some forms of epilepsy (Deransart et al., 1996; Deransart et al., 1998; Vercueil et al., 1998; Deransart et al., 1999). Thus, it is conceivable that inhibition of excitatory transmission at the STN-SNr synapse with group II mGluR agonists could

have broader therapeutic potential than L-DOPA and other drugs used for dopamine replacement in PD patients.

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Figure 1. Activation of group II mGluRs reduces EPSCs at the STN-SNr synapse. (A) Evoked EPSCs before (control), during (LY354740), and after (wash out) application of LY354740. Applications of LY354740 dramatically reduce EPSCs, and this effect is completely reversible. (B) Dose-response relationship of LY354740-induced inhibition of EPSCs. The effect of inhibition of EPSCs is maximal at 100 nM. Each point represents the mean of 3 experiments. (C) Effects of specific group II mGluR agonists on EPSCs at the STN-SNr synapse. Agonists include LY354740 (100 nM), APDC (100 μM), and DCG-IV (3 μM). Each bar represents the mean (± SEM) of data collected from 5 cells.

Figure 2. The LY354740-induced inhibition of EPSCs can be blocked by application of group II mGluR antagonists prior to application of the agonist. (A) Representative traces demonstrating the block of the effect of LY354740 on EPSC amplitude by the mGluR antagonist LY341495, and CPPG. (B) Time course of the effect of LY354740 on EPSC amplitude in the presence of LY341495. (C) Bar graphs showing the mean effect of LY354740 on EPSCs in the absence and presence of LY341495 (100 nM) or CPPG (500 μM). Each bar represents the mean (± SEM) of data collected from 5 cells. (*$p<0.01$).

Figure 3. Group II mGluRs are presynaptically localized at assymetric terminals in the SNr. (A-D) Electron micrograph demonstrating presynaptic mGluR2/3 immunoreactivity at assymetric terminals in SNr: Labeled (*) axon terminals (t) are shown synapsing on unlabeled dendrites (d) and dendritic spines (s). (E) An example of a labeled terminal forming a symmetric synapse . Synapses are indicated by arrows. [Bar = 301 nM (A); 203 nM (B); 315 nM (C); 263 nM (D); 207

nM (E)]

Figure 4. Activation of group II mGluRs has no effect on response to exogenously applied kainate. (A) Representative traces of Kainate-evoked currents in the SNr projection neurons before (control) and during application of 100 nM LY354740. (B) Time course of the effect of LY354740 on the amplitude of kainate-evoked currents. (C) Mean data demonstrating the lack of effect of group II mGluR activation on kainate-evoked currents. Mean ± SEM, $p>0.05$, $n=5$.

Figure 5. Inhibition of EPSCs at the STN-SNr synapses is mediated by a presynaptic mechanism. (A) Examples of mEPSCs before (pre-drug) and during application of the 100 nM LY354740. (B) Overlayed averages of all mEPSCs recorded before and during LY354740 application demonstrating the lack of effect on mEPSCs amplitude and kinetics. (C) Amplitude histograms of mEPSCs before (left) and during application of 100 nM LY354740 (D) Cumulative frequency plots illustrating the lack of effect of LY354740 on mEPSC amplitude (left) and inter-event interval (right) (Kolmogorov-Smirnov test; $p=0.99$). Data shown are representative of 5 separate experiments.

Figure 6. Activation of groupII mGluRs has no effect on inhibitory transmission in the SNr. (A) representative traces of evoked IPSCs before (Pre-Drug) and during the application of 100 nM LY354740. (B) Time course of the effect of LY354740 on IPSC amplitude. (C) Mean data demonstrating the lack of effect of group II mGluR activation on IPSC amplitude. Data represents mean ± SEM of 7 separate experiments ($p>0.05$).

Figure 7. Activation of group II mGluRs reverses catalepsy in an animal model of Parkinson's disease. Degree of haloperidol-induced catalepsy was measured as either latency to remove a paw from a bar (A) or latency to first paw movement when the animal was placed on a vertical grid (B). Haloperidol (1.5 mg/kg I.P.) induces a pronounced catalepsy which was reversed in a dose dependent manner by LY354740 (3-30 mg/kg I.P.) (* $p<0.05$). LY354740 alone had no effect on either measure of catalepsy. Data shown represent mean ±SEM of data collected from 8 animals.

Figure 1
Bradley et al., 1999

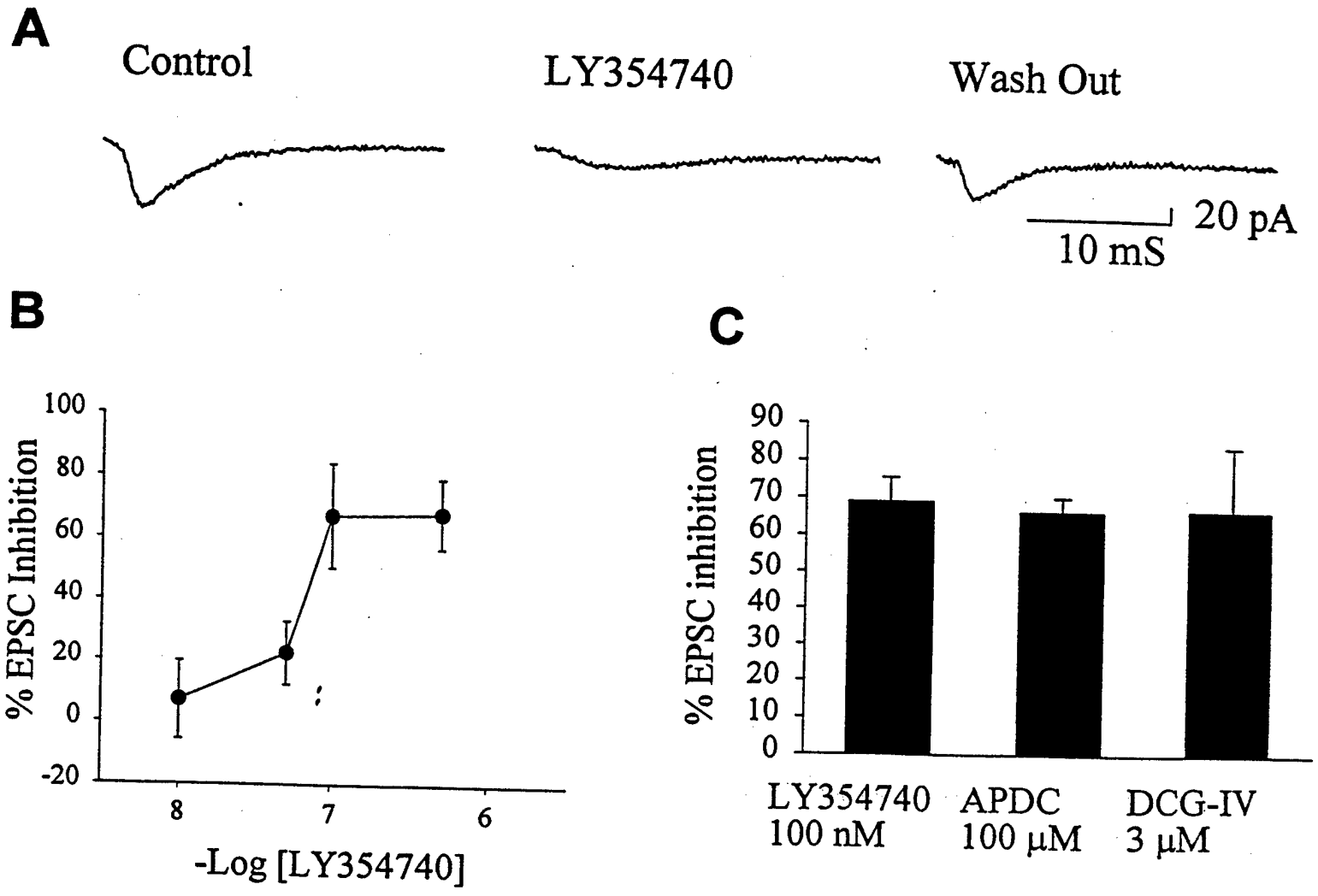

Figure 2
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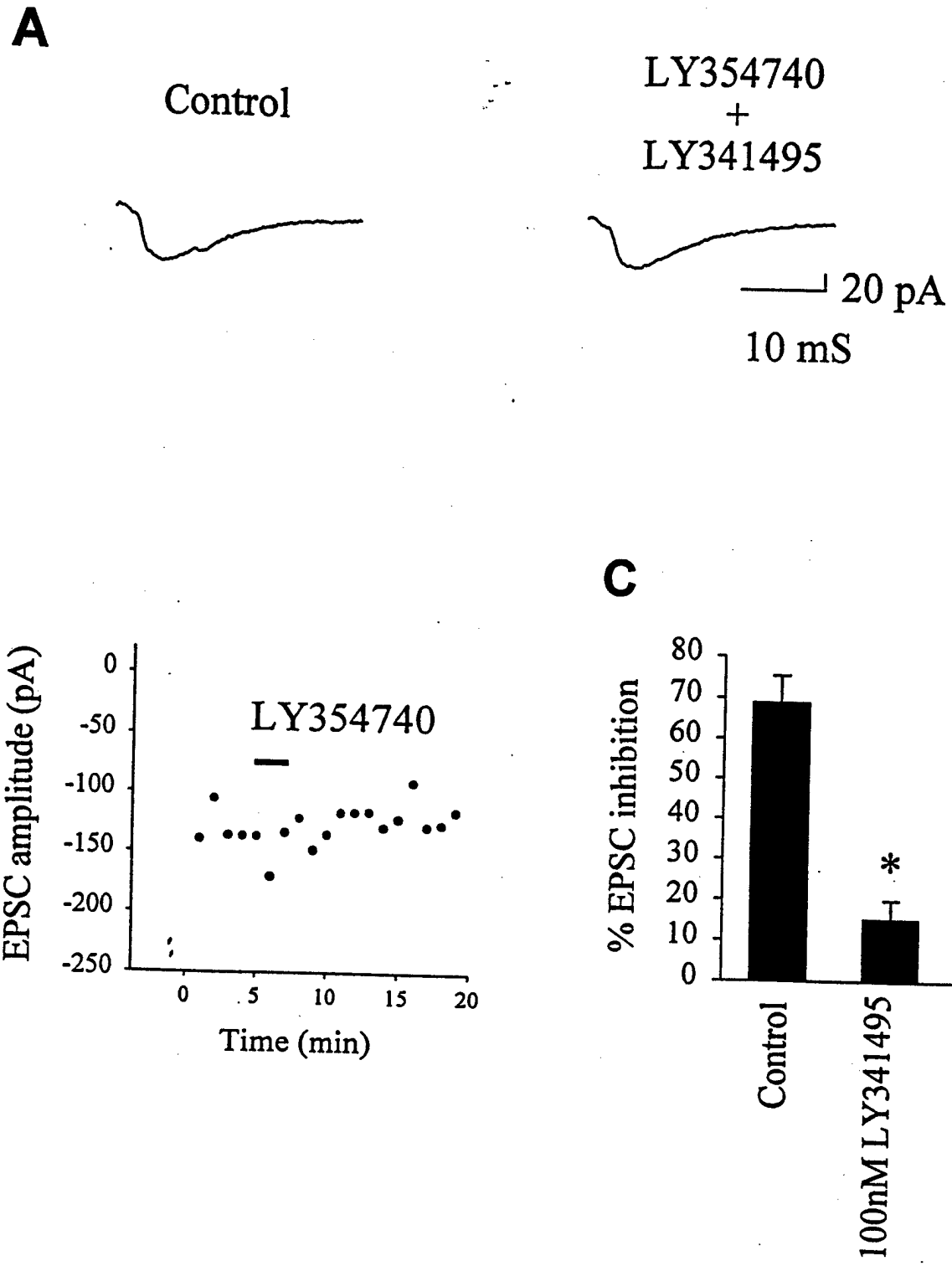

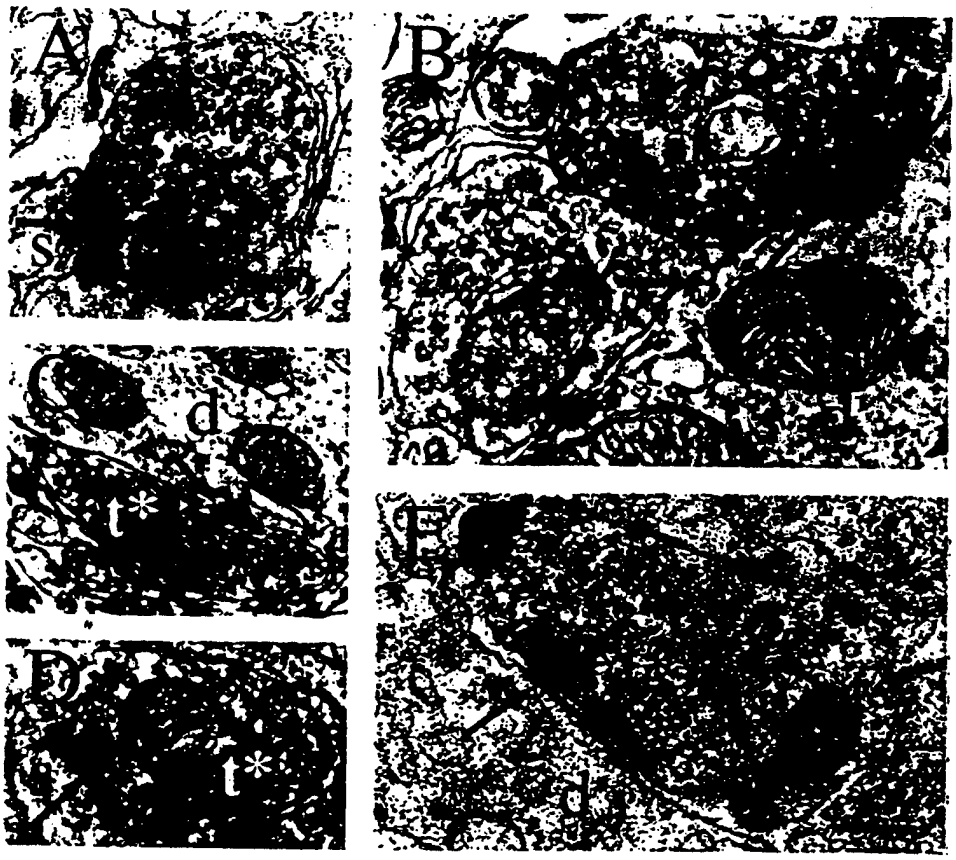

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Figure 3

Figure 4
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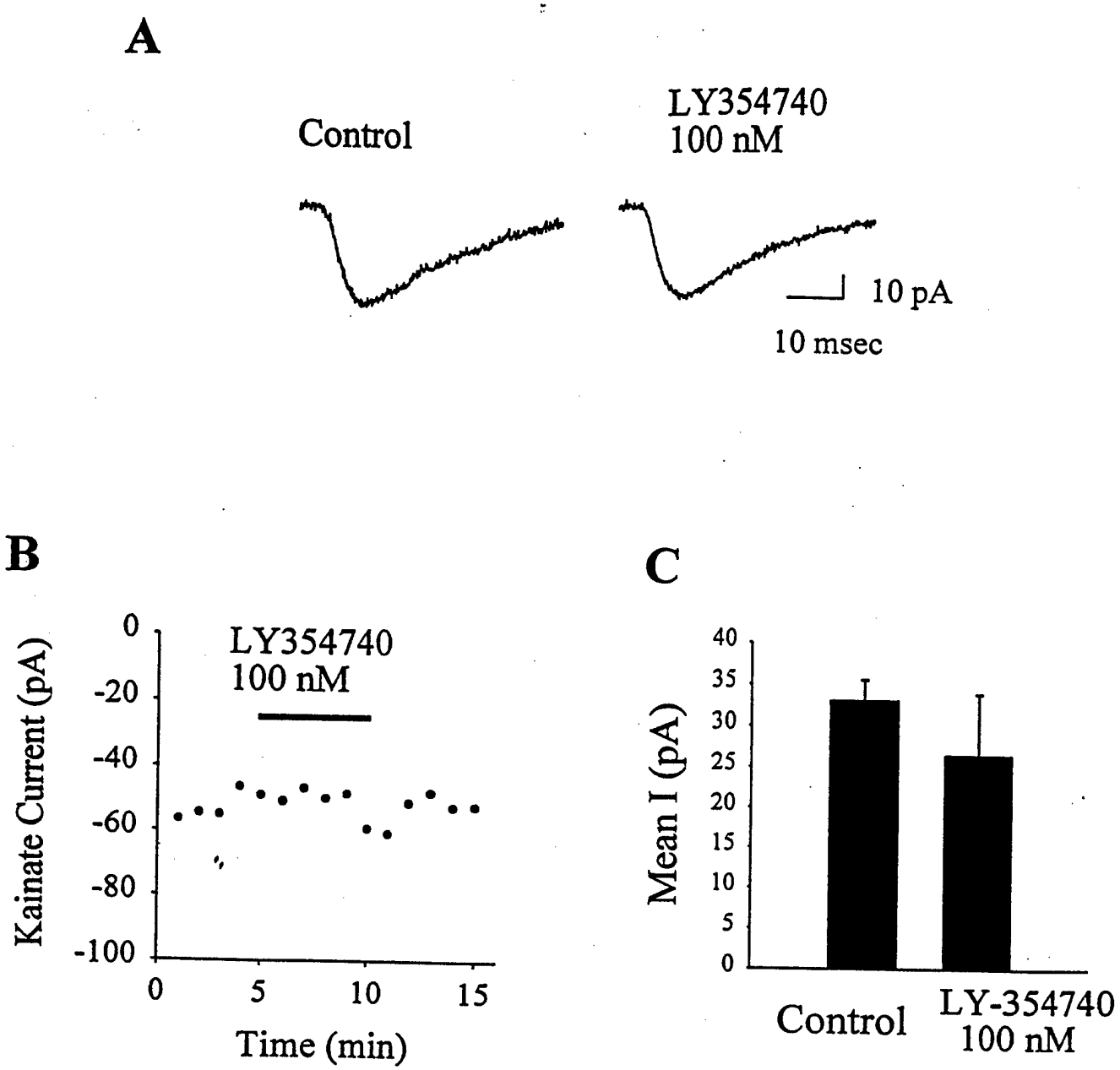

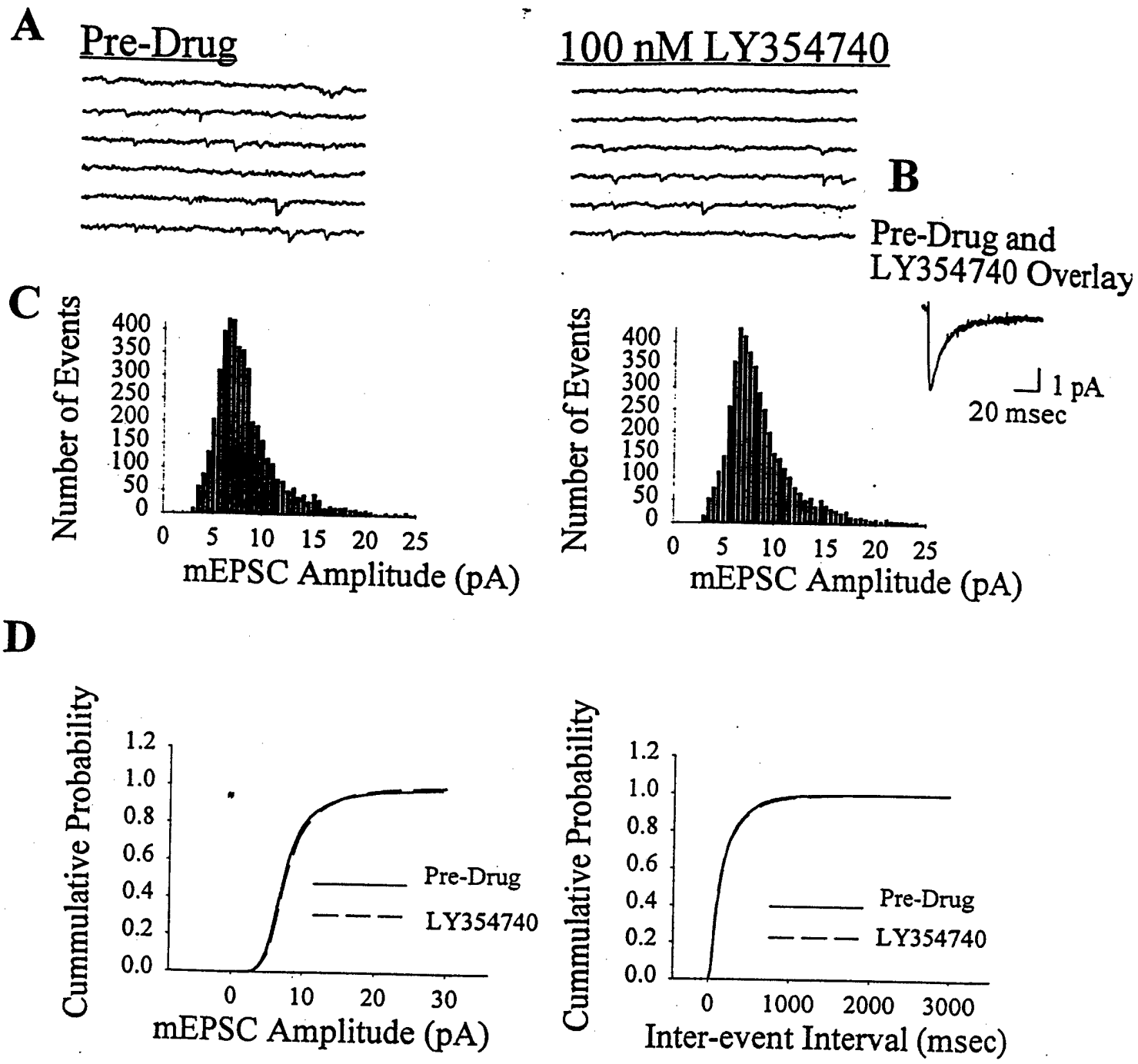
A
Pre-Drug
100 nM LY354740
B
Pre-Drug and
LY354740 Overlay
1 pA
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C
Number of Events
400
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D
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Figure 6
Bradley et al., 199

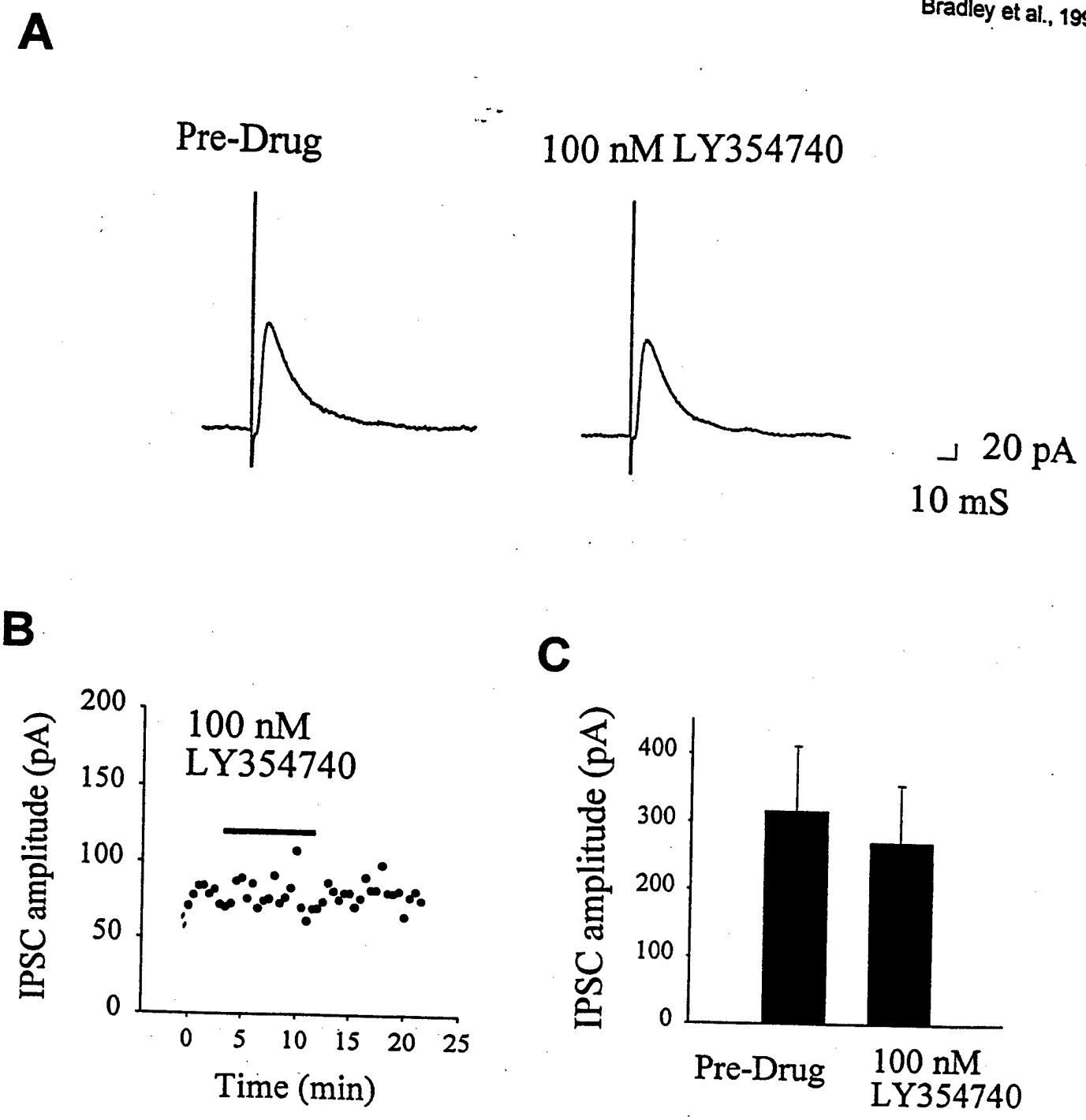
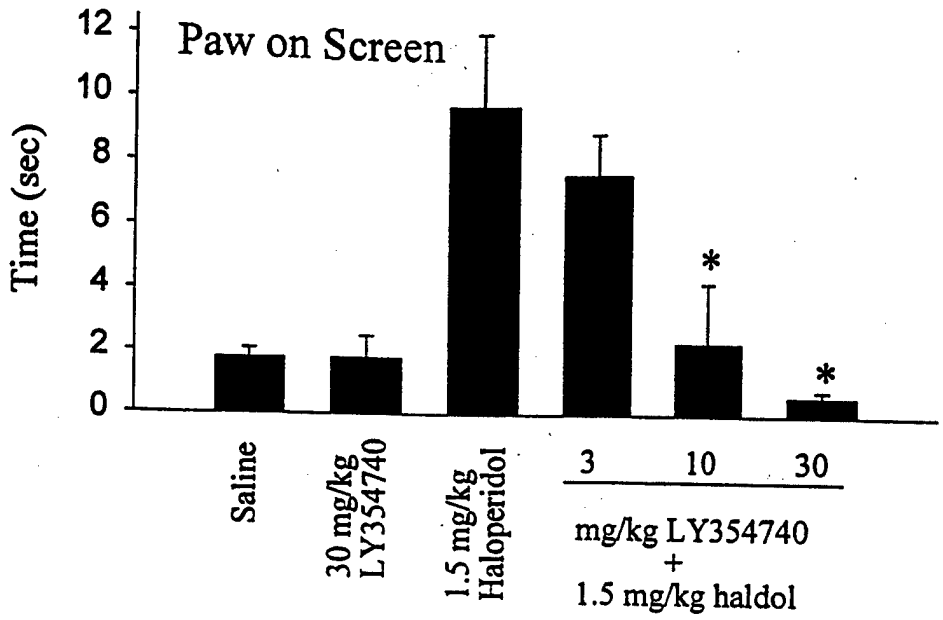


Figure 7
Bradley et al., 1999

A



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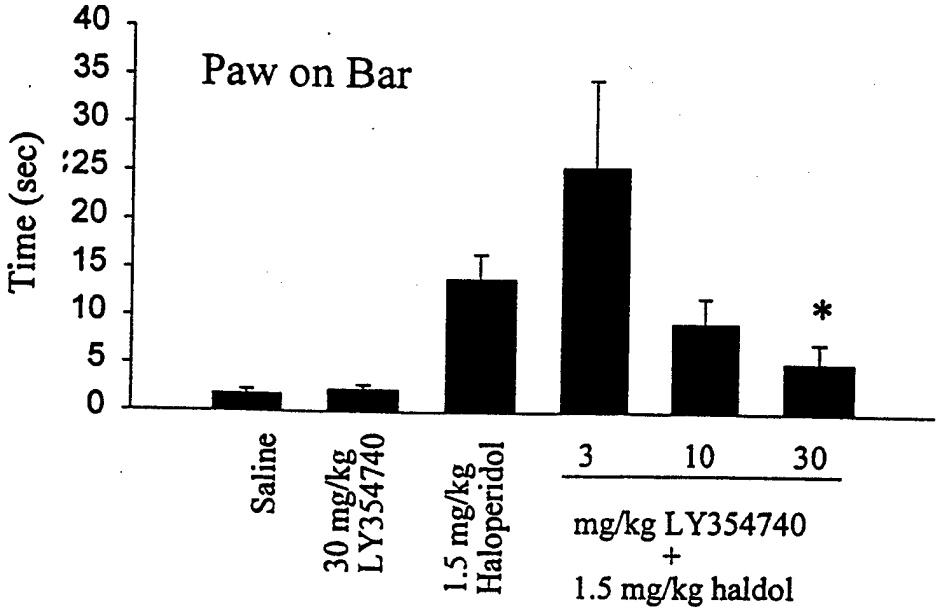

Activation of the Group I Metabotropic Glutamate Receptor mGluR1 Produces a Direct Excitation and Mediates a Slow EPSP in the Substantia Nigra Pars Reticulata.

Michael J. Marino, Stefania R. Bradley, Marion Wittmann[†], George M. Hubert[‡], Yoland Smith[‡], Allan I. Levey[‡], and P. Jeffrey Conn.

Departments of Pharmacology, and [‡]Neurology, Emory University, Atlanta, GA 30322 and [†] Tierphysiologie, University of Tuebingen, D-72076 Tuebingen, Germany.

Running title: Group I mGluRs in Substantia Nigra

Address Correspondences to: Dr. P.J. Conn

Department of Pharmacology

Rollins Research Building

Atlanta, GA 30322

(404) 727-5991

Fax: (404) 727-0365

email: pconn@emory.edu

SUMMARY

1. We present a combined electrophysiological and anatomical study of the direct excitatory effect of metabotropic gluatamate receptor (mGluR) activation on GABAergic neurons of the substantia nigra pars reticulata (SNr).

2. Activation of group I, but not group II or III mGluRs induces a robust depolarization of SNr GABAergic neurons. This depolarization results in an increase in firing frequency with potential implications for physiological and pathophysiological function of the SNr.

3. The depolarization is correlated with a decrease in membrane conductance, and the resulting inward current exhibits a current voltage relationship consistent with the inhibition of a leak potassium conductance.

4. Light level and electron microscopic examination of mGluR1 and mGluR5 immunoreactivity indicate that both group I mGluRs are postsynaptically localized at symmetric and assymetric terminals in the SNr.

5. Interestingly, a pharmacological characterization using subtype selective agonists and antagonists indicates that mGluR1 is the sole mediator of this depolarization. The depolarizing actions of the group I selective agonist DHPG (100 μM) is antagonized by the mGluR1 selective antagonist CCPCOEt (100 μM) but not by the mGluR5 selective antagonists MPEP (10 μM). Furthermore, the effect of DHPG is not mimicked by the mGluR5 selective agonist CBPG (100 μM).

6. High frequency stimulation of SNr afferents in the presence of antagonists of ionotropic glutamate receptors and GABA receptors induces a slow EPSP in SNr GABAergic

neurons. This slow EPSP exceeds action potential threshold and induces firing in these cells. Application of the mGluR1 selective antagonist CCPCOEt reversibly blocks the slow EPSP indicating that synaptically released glutamate acts on mGluR1 receptors in SNr projection neurons to modulate excitability.

INTRODUCTION

The basal ganglia are a richly interconnected group of subcortical nuclei involved in the control of motor behavior. The primary input nucleus of the basal ganglia is the striatum, and the primary output nuclei are the substantia nigra pars reticulata (SNr) and the globus pallidus internal capsule (entopeduncular nucleus in rodents). The striatum projects to these output nuclei both directly, providing an inhibitory GABAergic input, and indirectly through the globus pallidus external segment and the subthalamic nucleus (STN) providing an excitatory glutamatergic input. the balance between these two inputs is believed to be of critical importance in the control of motor behavior, and disruptions in this balance are believed to underlie a variety of movement disorders (Wichmann & DeLong, 1997;Wichmann & DeLong, 1998)

While much effort has been directed at elucidating the basic connectivity of the direct and indirect pathway, less is known about the modulatory influence various transmitters may have in these structures. In particular, the role of glutamate as a modulator acting on metabotropic glutamate receptors (mGluRs) in the SNr holds much promise both for treatment of these debilitating neurological disorders as well as for enhancing our understanding of the role the SNr plays in normal brain function. To date, eight mGluR subtypes (mGluR1-8) have been cloned, and are classified into 3 major groups based on sequence homology, coupling to second messenger systems, and selectivities for various agonists (see (Conn & Pin, 1997) for review). Group I mGluRs (mGluR1, and 5) couple to phosphoinositide turnover, while groups II (mGluR2, and 3) and III (mGluR4, 6, 7, and 8) couple to the inhibition of adenylate cyclase. These metabotropic glutamate receptors(with the exception of mGluR6, and 8) are widely distributed throughout the central nervous system where they play important roles in regulating

cell excitability and synaptic transmission.

Previous studies have shown that mGluRs are highly expressed throughout the basal ganglia (Kerner *et al.*, 1997a;Testa *et al.*, 1994;Bradley *et al.*, 1999a;Kosinski *et al.*, 1999;Bradley *et al.*, 1999b;Kerner *et al.*, 1997b;Kosinski *et al.*, 1998;Testa *et al.*, 1998), and play important roles in the regulation synaptic transmission in the SNr. Group II and III mGluRs mediate a presynaptic inhibition of excitatory transmission at the STN-SNr synapse (Bradley *et al.*, 1999c), while group I and III mGluRs mediate inhibition of GABAergic transmission in the SNr (Wittman *et al.*, 1999). One of the predominate postsynaptic effects of mGluRs is a group I mGluR-mediated depolarization observed in a variety of brain regions (Guerineau *et al.*, 1995;Guerineau *et al.*, 1994;Crepel *et al.*, 1994;Miller *et al.*, 1995). Since glutamatergic innervation of the SNr plays an important role in motor control, an understanding of the role postsynaptic mGluRs may play in modulating the activity of SNr GABAergic neurons could provide crucial insight on both the physiology and pathophysiology of the SNr. We now present a combined anatomical and electrophysiological study of the role of postsynaptic mGluRs in SNr projection neurons. Our findings demonstrate that activation of the postsynaptically localized group I mGluR mGluR1 mediates a pronounced increase in SNr GABAergic neuron excitability. Furthermore, we demonstrate that synaptically released glutamate acting on mGluR1 produces a slow EPSP capable of modulating the firing rate of SNr neurons.

METHODS

Materials

[R-(R*,S*)]-6-(5,6,7,8-Tetrahydro-6-methyl-1,3-dioxolo[4,5-g]isoquinolin-5-yl)furo[3,4-e]-1,3-benzodioxol-8(6H)-one (Bicuculline), 6-Cyano-7-nitroquinoxaline-2,3-dione (CNQX), D(-)-2-Amino-5-phosphonopentanoic acid (D-AP5), (RS)-3,5-Dihydroxyphenylglycine (DHPG), (RS)-3-Amino-2-(4-chlorophenyl)-2-hydroxypropyl-sulphonic acid (2-Hydroxysaclofen), and L(+)-2-Amino-4-phosphonobutyric acid (L-AP4) were obtained from Tocris (Ballwin, MO). (S)-(+)-2-(3'-carboxy-bicyclo[1.1.1]pentyl-)glycine (CBPG) was obtained from Alexis Corp. (San Diego, CA). (+)-2-aminobicyclo[3.1.0]-hexane-2,6-dicarboxylate monohydrate (LY354740) was a gift from D. Schoepp and J. Monn (Eli Lilly, Indianapolis, IN). Methylphenylethynylpyridine (MPEP), and 7-hydroxyiminocyclopropan-[b]chromen-1a-carboxylic acid ethyl ester (CCPCOEt) were gifts from R. Kuhn (Novartis, Basel, Switzerland). All other materials were obtained from Sigma (St. Louis, MO).

Electrophysiology

Whole-patch clamp recordings were obtained as previously described(Marino *et al.*, 1998) except that recordings were made under visual control. 15-18 day old Sprague-Dawley rats were used for all patch clamp studies. Brains were rapidly removed and submerged in an ice cold sucrose buffer (in mM: Sucrose, 187; KCL, 3; $MgSO_4$, 1.9; KH_2PO_4, 1.2; Glucose, 20; $NaHCO_3$, 26; equilibrated with 95% O_2/5% CO_2). Parasaggital, or horizontal slices (300μM thick) were made using a Vibraslicer (WPI). Slices were transferred to a holding chamber

containing normal ACSF (in mM: NaCl, 124; KCL, 2.5; $MgSO_4$, 1.3; NaH_2PO_4,1.0; $CaCl_2$, 2.0; Glucose, 20; $NaHCO_3$, 26; equilibrated with 95% O_2/5% CO_2). In some experiments, 5 μM glutathione, 500 μM pyruvate, and 250 μM kynurenate were included in the sucrose buffer and holding chamber. These additional compounds tended to increase slice viability but did not have any effect on experimental outcome. Therefore all of the data from these two groups have been pooled. Slices were transferred to the stage of a Hoffman modulation contrast microscope and continually perfused with room temperature ACSF (~3 mL/min, 23-24°C). Neurons in the substantia nigra pars reticulata were visualized with a 40X water immersion lens. Patch electrodes were pulled from borosilicate glass on a Narashige vertical patch pipette puller and filled with (in mM: potassium gluconate, 140; HEPES, 10: NaCl, 10; EGTA, 0.6; GTP, 0.2; ATP, 2; pH adjusted to 7.5 with 0.5 N NaOH). Biocytin (0.5%, free base) was added just prior to use. Electrode resistance was 3-7 MΩ. For measurement of synaptically evoked slow EPSPs, bipolar tungsten electrodes were used to apply stimuli to the SNr approximately 100 μm rostral to the recording site. Slow EPSPs were evoked in the presence of 10 μM CNQX, 10 μM D-AP5, 10 μM bicuculline, and 100 μM 2-hydroxy-saclofen.

Immunocytochemistry

Preparation of the tissue for immunocytochemistry analysis at electron microscopy level was obtained following published protocols(Bradley *et al.*, 1996). The avidin-biotin-peroxidase method (Vectastain Elite ABC kit, Vector laboratories, Burlingame, CA) was used to detect mGluR2/3 immunoreactivity in rat (n=2) SNr. The peroxidase reaction was developed in 0.05% DAB and 0.01% H_2O_2. Commercially available antibodies were employed that specifically recognize mGluR1 (Chemicon, Temecula, CA) and mGluR5 (Upstate, Lake Placid, NY).

RESULTS

Identification of GABAergic neurons in the SNr. While the SNr is largely composed of GABAergic projection neurons, there are a significant number of "displaced" dopaminergic neurons within the nucleus (Richards *et al.*, 1997;Nakanishi *et al.*, 1987;Hausser *et al.*, 1995). These two neuronal types exhibit distinct electrophysiological and morphological features. We therefore took measures to positively identify all neurons used in these studies as GABAergic. As shown in figure 1, GABAergic neurons exhibited spontaneous repetitive firings, short duration action potentials (half amplitude duration = 1.7 ± 0.2 msec), little spike accommodation, and a lack of inward rectification, while dopaminergic neurons displayed no, or low frequency spontaneous firings, longer duration action potentials (half amplitude duration = 7.0 ± 0.5 msec), strong spike accommodation, and a pronounced inward rectification. Light microscopic examination of biocytin filled neurons indicated that GABAergic neurons had extensive dendritic arborizations close to the cell body, whereas dopaminergic neurons had sparser dendritic structure (data not shown). All of the data presented in these studies are from confirmed GABAergic neurons.

Activation of group I mGluRs depolarizes SNr GABAergic neurons. Previous studies have demonstrated that all three groups of mGluRs are expressed in the SNr (Testa *et al.*, 1994;Testa *et al.*, 1998). We therefore employed maximal concentrations of highly specific group-selective mGluR agonists to determine in activation of these receptors had any effect on membrane properties of Snr GABAergic neurons. In the presence of 0.5 μM tetrodotoxin (TTX),

application of the group I mGluR-selective agonist DHPG induces a robust direct depolarization (300 μM DHPG, 16.1 ± 2.6 mV) of SNr neurons which reverses upon drug washout (Fig 2A,C). This depolarization is accompanied by a significant increase in input resistance (pre-drug, 498 ± 70 MΩ; 100 μM DHPG, 619 ± 89 MΩ; $p<0.05$ paired t) (Fig 2B) suggesting that a DHPG-induced decrease in membrane conductance underlies this effect. The concentration-response relationship for DHPG-induced depolarization of SNR GABAergic neurons exhibited an EC_{50} of approximately 40 μM, consistent with an effect on group I mGluRs (Schoepp *et al.*, 1994). In contrast to this group I mGluR-mediated depolarization, the group II selective LY354740 (Monn *et al.*, 1997;Kingston *et al.*, 1998) and the group III selective L-AP4 (Conn & Pin, 1997)had no significant effect on resting membrane potential (Fig 2A-C). We therefore focused on the physiology and pharmacology of this group I mGluR-mediated depolarization.

Group I mGluR activation causes an increase in action potential firing in SNr GABAergic neurons. To determine the effect of group I mGluR activation on action potential firing in SNr GABAergic neurons, we applied DHPG in the absence of TTX. At the beginning of whole cell recording cells fire spontaneous action potentials (Fig 1) however, within a few minutes, cells tend to hyperpolarize and do not fire spontaneously when the membrane potential is maintained at -65 mV by current injection. Application of 100 μM DHPG induces a robust depolarization, and a large increase in action potential firing (Fig 3A). This DHPG-induced spiking is completely blocked by injection of hyperpolarizing current to maintain a -65 mV membrane potential during drug application, and is mimicked by direct depolarization of the cells to the same membrane potential (100 μM DHPG, 3.8 ± 0.3 Hz; direct depolarization 3.2 ±

0.7 Hz, $p>0.05$ students t). Interestingly, cells held at -65 mV by current injection exhibited a significant shift in the relationship between amplitude of depolarizing current injection and spike frequency (repeated measures ANOVA, $p<0.01$)(Fig 3B,C). This is consistent with the approximate 20% increase in input resistance observed in the presence of TTX (Fig 2B) which would be expected to enhance the depolarization produced by current injection. DHPG had no effect on spike amplitude (pre-drug 72.7 ± 5.1 mV; DHPG 70.7 ± 7.7 mV; n=3, $p>0.05$), or half amplitude spike width (pre-drug 1.6 ± 0.2 ms; DHPG 1.7 ± 0.5 mS; n=3, $p>0.05$).

The DHPG-induced excitation of SNr GABAergic neurons is mediated by the inibition of a leak potassium conductance. In other neurons, activation group I mGluRs has been demonstrated to depolarize the cells by inhibition of a leak potassium conductance (Guerineau *et al.*, 1994), or by an increase in a non-selective cationic conductance (Guerineau *et al.*, 1995;Miller *et al.*, 1995). Our observation that DHPG causes an increase in input resistance indicates that an inhibition of leak potassium conductance is the most likely mechanism underlying this effect. We directly tested this hypothesis in a series of voltage clamp experiments in which holding potential was ramped between -40 and -120 mV (20 mV/s) to establish a current-voltage relationship. Application of 100 μM DHPG induces an inward shift in holding current (Fig 4A) which is evident as a change in the slope of the whole cell current-voltage relationship (Fig 4B). Subtracting the pre-drug I-V trace from the trace in the presence of DHPG reveals a near linear I-V relationship for the DHPG-induced current. which reverses near the calculated potassium equilibrium potential (-103.4 mV) (Fig 4C). Binning the data in 10 mV segments and averaging over 5 independent experiments produces a current voltage relationship

which is well fit by a straight line (r=0.995) and has an interpolated reversal potential of 108.8 ± 8.5 mV, in agreement with the calculated potassium equilibrium potential (Fig 4D). Taken together, these data strongly support the hypothesis that the DHPG-induced depolarization of SNr GABAergic neurons is mediated by decreasing a leak potassium conductance.

Group I mGluRs are postsynaptically localized in SNr neurons. DHPG activates both mGluR1 and mGluR5. In order to determine if both of these receptors are localized at postsynaptic sites in SNr neurons, we performed immunocytochemical studies with antibodies selective for mGluR1a, and mGluR5.

At the light microscopic level, the SNr exhibited labeling for both mGluR1a and mGluR5 (Fig 5,6). In order to determine the pre- or postsynaptic nature of this labeling, we employed electron microscopy. Both antibodies primarily labeled dendritic processes of symmetric and assymetric terminals as well as a few glial processes (Fig 5,6). While the majority of labelling was postsynaptic, it should be noted that mGluR1a immunoreactivity was also found in small unmylenated axons and a few axon terminals.

The DHPG-induced excitation of SNr GABAergic neurons is mediated by mGluR1. Our findings that both mGluR1 and mGluR5 are postsynaptically localized in SNr projection neurons suggests that both of these receptors could be involved in the DHPG-induced depolarization. In order to determine the role each of these receptors plays in this effect we employed newly available pharmacological tools that distinguish between mGluR1 and mGluR5. CBPG, a partial agonist at mGluR5 which has antagonistic properties at mGluR1 (Yokoi *et al.*,

1996;Mannaioni *et al.*, 1999) failed to induce a depolarization at maximal concentrations (Fig 7), indicating that the depolarizing effect of DHPG is likely due to activation of mGluR1. Consistent with this, pretreatment with the highly selective, noncompetitive mGluR1 antagonist CCPCOEt (Annoura *et al.*, 1996;Casabona *et al.*, 1997;Litschig *et al.*, 1999) produced a significant reduction in the DHPG-induced depolarization of SNr GABAergic neurons, while pretreatment with MPEP, a highly selective, noncompetitive antagonist of mGluR5 had no significant effect at concentrations shown the be effective at blocking mGluR5 in other systems (Bowes *et al.*, 1999;Gasparini *et al.*, 1999) (Fig 7).

mGluR1 mediates a slow EPSP in SNr GABAergic neurons. The data presented thus far indicate that mGluR1 mediates direct excitation of SNr projection neurons. The SNr receives a sparse yet important glutamatergic innervation from the subthalamic nucleus (STN), and burst firing of the STN is known to play a key role in several neurological disorders including Parkinson's disease (Hollerman & Grace, 1992;Bergman *et al.*, 1994;Hassani *et al.*, 1996)and epilepsy (Deransart *et al.*, 1996;Deransart *et al.*, 1998;Deransart *et al.*, 1999). If activation of glutamatergic afferents to the SNr release sufficient glutamate to activate mGluR1 receptors, the resulting excitation of SNr projection neurons could play an important role in these disease states. We tested this hypothesis by recording from SNr GABAergic neurons in the presence of ionotropic glutamate receptor and GABA receptor antagonists. High frequency stimulation (25-50 Hz, 100 μS) of the afferents within the SNr produced a robust and reliable slow EPSP which reached threshold for action potential firing in 4 of 4 cells (Fig 8). Consistent with a mediation by mGluR1, this slow EPSP was completely and reversibly blocked by 100 μM CCPCOEt.

DISCUSSION

The data presented here demonstrate that activation of postsynaptically localized group I mGluRs in SNr GABAergic neurons produces a decrease in membrane conductance, likely mediated by the inhibition of a leak potassium channel. This change in membrane conductance underlies a group I mGluR-mediated depolarization and increase in action potential firing. Furthermore, this effect is due to selective activation of mGluR1, and can be produced by synaptically released glutamate.

Our immunocytochemical studies revealed both mGluR1 and mGluR5 are present in SNr projection neurons. Interestingly, our pharmacological studies demonstrate that activation of mGluR1 is solely responsibly for the group I-mediated depolarization. This is of interest since both mGluR1 and mGluR5 couple to phosphoinositide turnover, and are capable of inducing depolarization in a variety of brain regions (see (Conn & Pin, 1997;Anwyl, 1999)for review). Thus, there must be some degree of specificity produced by subsynaptic localization or some other functional segregation of these receptors. It should be noted that while mGluR1 plays the predominate role in mediating the group I mGluR-induced depolarization in SNr, mGluR5 may play important physiological roles regulating cell properties which were not measured in these studies. For example, group I mGluRs are known to modulate NMDA receptor currents in a variety of brain regions, and it is possible that mGluR5 is involved in a similar modualtion in SNr. Future studies on the role of mGluR5 in these cells may provide important insight into the functional roles of closely related receptor subtypes within a single neuronal population.

Our current findings add to a growing body of literature that suggests group I mGluRs play an important role in basal ganglia function. For instance, mGluR5 is heavily expressed in

striatum, and is also present in subthalamic nucleus, and at lower levels in the pallidal complex (Testa *et al.*, 1994;Testa *et al.*, 1995;Kerner *et al.*, 1997b;Tallaksen-Greene *et al.*, 1998). While the levels of mGluR1 are more limited, this receptor is also expressed throughout the basal ganglia(Testa *et al.*, 1994;Kerner *et al.*, 1997b;Tallaksen-Greene *et al.*, 1998). A number of studies suggests that agonists of group I mGluRs may act at several levels to increase the net activity of projection neurons in the BG output nuclei. For instance, activation of group I mGluRs potentiates NMDA receptor currents in striatal neurons(Pisani *et al.*, 1997;Colwell & Levine, 1994) and behavioral studies combined with studies of changes in 2-deoxyglucose uptake and fos immunoreactivity suggest that injection of group I mGluR agonists in the striatum induces a selective activation of the indirect pathway from the striatum to the output nuclei(Kearney *et al.*, 1997;Kaatz & Albin, 1995) . Furthermore, group I mGluRs are present in STN and recent physiological studies suggest that activation of these receptors has profound excitatory effects in STN projection neurons(Abbott *et al.*, 1997;Awad & Conn, 1999). Therefore, group I mGluRs exert excitatory influence at multiple levels throught the basal ganglia, and insight into the group I mGluR subtypes mediating these effects may have important implications for development of pharmacological treatments of basal ganglia disorders.

The finding that activation of mGluR1 by synaptically released glutamate induces a slow EPSP in SNr GABAergic neurons is of particular interest for understanding the role the STN plays in modulation of the SNr. Approximately 90% of synaptic input on SNr projection neurons is composed of inhibitory GABAergic projections forming the so called direct pathway (Smith *et al.*, 1998). The indirect pathway is composed of striatal projections through the globus pallidus and the STN which constitute a large percentage of the excitatory terminals on SNr GABAergic

neurons (Smith *et al.*, 1998). While the glutamatergic input to the SNr is sparse, it plays a critical role in basal ganglia function as evidenced by the pronounced clinical effects of STN lesions (Guridi & Obeso, 1997). The STN also plays a key role in pathological activity of the SNr. Transition of STN neurons from single spike activity to burst-firing mode, and resultant over excitation of the SNr is implicated in Parkinson's disease (Hollerman & Grace, 1992;Bergman *et al.*, 1994;Hassani *et al.*, 1996) as well as some forms of epilepsy (Deransart *et al.*, 1996;Deransart *et al.*, 1998;Deransart *et al.*, 1999). Furthermore, STN neurons exhibit extremely high firing rates, and can typically exceed 25-50 Hz during burst-firing mode (Bergman *et al.*, 1994;Wichmann *et al.*, 1994;Beurrier *et al.*, 1999;Bevan & Wilson, 1999;Hollerman & Grace, 1992). In light of our current findings, it is likely that one of the effects of burst-firing of the STN is an activation of mGluR1, and a resultant depolarization of SNr neurons. Furthermore, it is possible that mGluR1 activation plays a more subtle neuromodulatory role during normal basal ganglia function.

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FIGURE LEGENDS

Figure 1. Demonstration of the identification of SNr GABAergic neurons. (A) Response of a GABAergic (left) and dopaminergic (right) neuron to depolarizing and hyperpolarizing current injections. Note the pronounced spike frequency adaptation and inward rectification exhibited by the dopaminergic cell which is absent in the GABAergic cell. (B) Examples of spike activity from resting cells. GABAergic neurons (left) fire at high frequency, while dopaminergic neurons (right) exhibit lower frequency or no spontaneous activity. (C) comparison of single action potentials from a GABAergic (left) and dopaminergic (right) neuron. All data presented here are from confirmed GABAergic neurons.

Figure 2. DHPG induces a group I mGluR-mediated depolarization of SNr projection neurons. (A) 100 μM DHPG induces a depolarization and (B) concominant increase in input resistance in SNr GABAergic neurons. Maximal concentrations of the group II-selective agonist LY345740, and the group III-selective agonist L-AP4 are without effect. (C) Mean ± SEM of data from 5 cells demonstrating that at maximal concentrations, only the group I agonist DHPG induces a depolarization. (D) Concentration-response relationship of the DHPG-induced depolarization.

Figure 3. Activation of group I mGluR increases spike firing in SNr GABAergic neurons. (A) Current clamp recording from a GABAergic neuron in the absence of TTX. At a resting mebrane potential of -65 mV, the cells are not spontaneously active. Application of 100 μM DHPG induces a robust depolarization, and high frquency action potential firing. (B) Cells in the

presence and absence of DHPG were maintained at a resting membrane potential of -65 mV by current injection. DHPG treated cells respond to depolarizing current pulses with a larger depolarization and higher frequency firing, consistent with an increase in input resistance. (C) Graph of spike frequency in response to depolarizing current injections from 5-100 pA from a resting membrane potential of -65 mV. In the presence of DHPG (open circles) cells fired at higher frequency than was observed prior to drug application (closed circles). Data represents the mean ± SEM of spike frequencys recorded from 3 separate cells. Spike frequency is normalized to the maximal pre-drug frequency in order to account for differences in basal responses.

Figure 4. Activation of group I mGluRs decreases a potassium conductance in SNr GABAergic neurons. (A) Application of 100 μM DHPG induces an inward shift in holding current which reverses on drug washout. (B) This inward shift is evident in the whole cell current-voltage relation determined by applying voltage ramps from -40 to -120 mV. (C) Subtraction reveals a linear current which is inward at normal resting potentials, and reverses near the predicted potassium equilibrium potential. (D) Mean ± SEM of data from 5 cells. The interpolated reversal potential is -108.8 ± 8.5 mV which compares favorably with the calculated potassium equilibrium potential of -103.4 mV.

Figure 5. The group I mGluR1a subunit is localized at postsynaptic sites within the SNr. (A) Low power light micrograph of mGluR1a immunostaining in the SNc and SNr. (B) High power light micrograph of mGluR1a immunoreactive processes in the SNr. Lightly labeled neuronal cell bodies are indicated by asterisks. (C) Low power electron micrograph of mGluR1a

immunoreactive dendrites (Den) in SNr. Note that the immunoreactivity is mostly found in dendritic processes but also occurs in small, unmylenated axons (Ax) and a few axon terminals (Te). (D-E) High power electron micrographs of mGluR1a-immunoreactive dendrites that form asymmetric (arrowheads) and symmetric(arrow) synapses with unlabeled terminals. (F) High power electron micrograph showing an mGluR1a-immunoreactive terminal in contact with a small, labeled dendrite. Note also the presence of an immunoreactive glial process (Gl) surrounding an unlabeled terminal. Scale bars: A: 500μm, B: 50 μm, C: 1 μm, D-F: 0.5 μm.

Figure 6. The group I mGluR5 subunit is localized at postsynaptic sites within the SNr. (A) Low power light micrograph of mGluR5 immunostaining on the SNc and SNr. (B) High power light micrograph of mGluR5-immunoreactive processes in the SNr. Labeled cell bodies are indicated by asterisks. (C) Low power electron micrograph of mGluR5-immunoreactive dendrites (Den) in the SNr. Note that the mGluR5 immunoreactivity is confined to dendritic processes. (D-E) High power electron micrograph of mGluR5-immunoreactive dendrites and spines (Sp) that form asymmetric synapses (arrowheads) with unlabeled terminals. Note the presence of an immunoreactive glial process (Gl). Scale bars: A: 500 μm, B: 50 μm, C: 1μm, D-E: 0.5 μm.

Figure 7. The group I mGluR induced depolarization is mediated by mGluR1. (A) representative traces demonstrating that the DHPG induced depolarization of SNr GABAergic neurons is not mimicked by the mGluR5 selective agonist CBPG. Furthermore, the highly selective noncompetitive mGluR1 antagonist CCPCOEt fully blocks the DHPG-induced depolarization, while the mGluR5 selective antagonist MPEP is without effect. (B) Mean ± SEM of data from 5

cells per condition demonstrating the selective antagonisim of the group I mGluR mediated depolarization of SNr projection neurons by the mGluR1 selective antagonist CCPCOEt. (*$p<0.01$ students t)

Figure 8. mGluR1 mediates a slow EPSP in SNr GABAergic neurons. High frequency stimulation of afferents in the SNr in the presence of ionotropic glutamate receptor and GABA receptor antagonists elicits a slow EPSP which exceeds action potential threshold and induces firing. This EPSP is fully and reversibly blocked by the selective mGluR1 antagonist CCPCOEt. Traces are representative of results obtained in four independent experiments.

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Figure 1

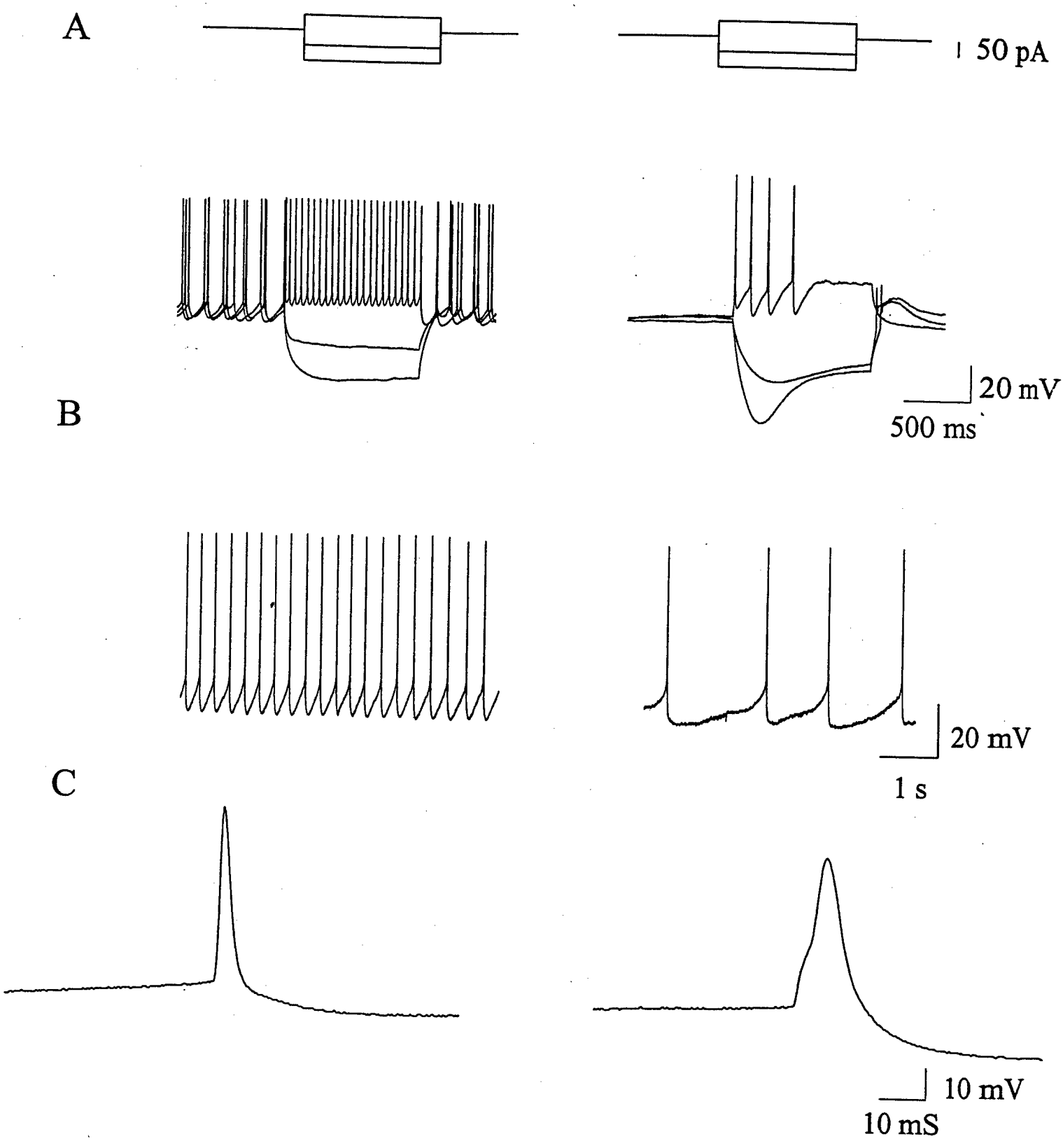

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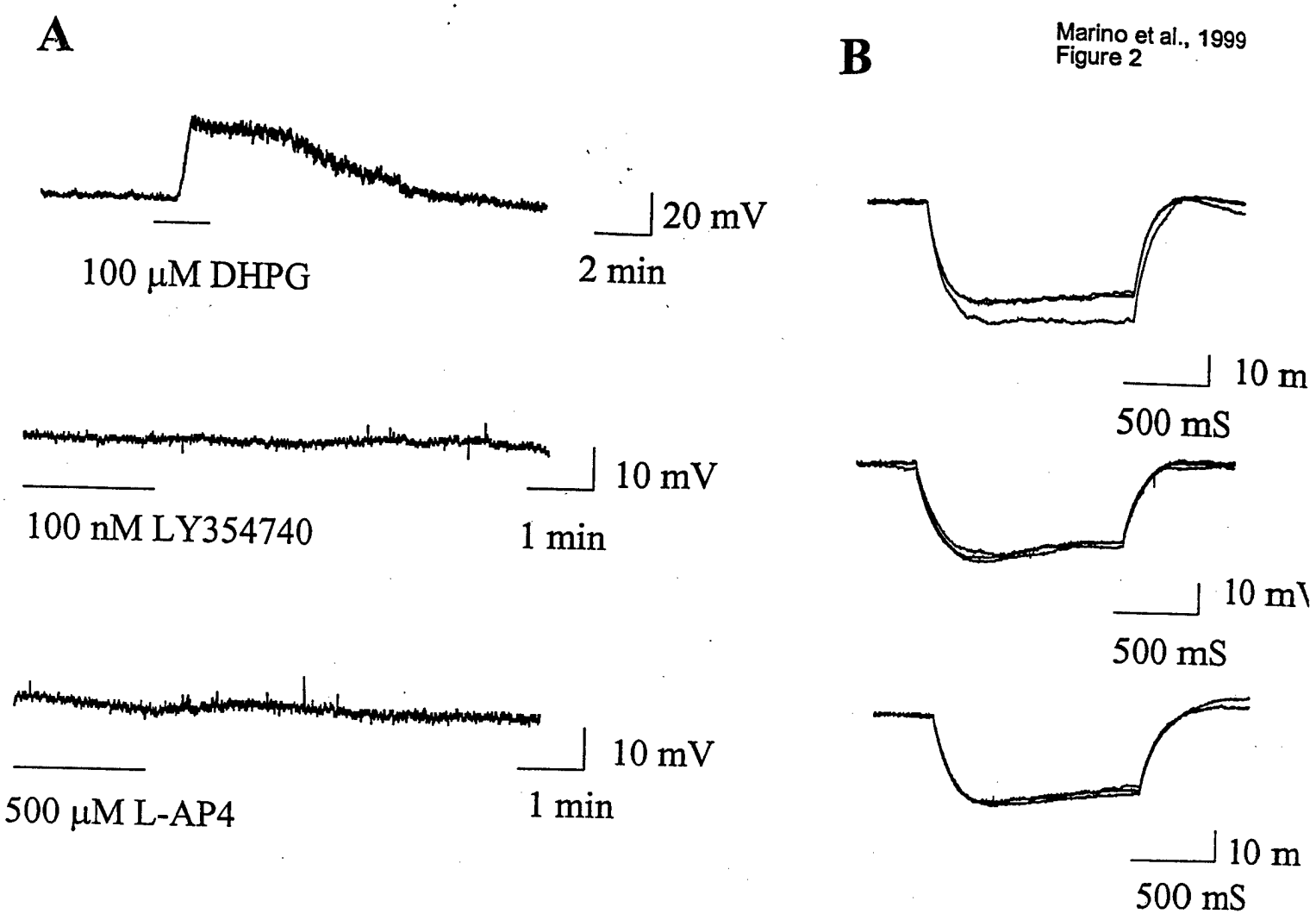
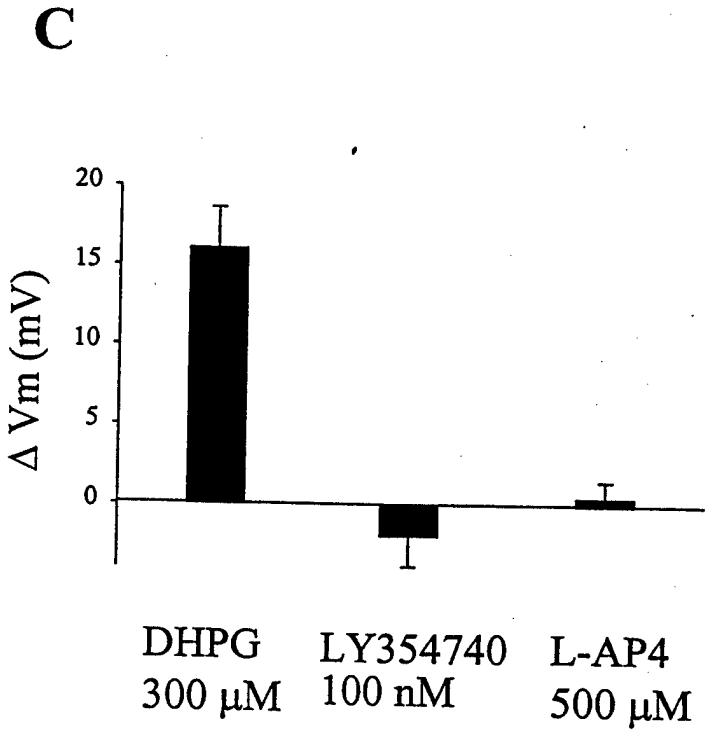
Figure 2





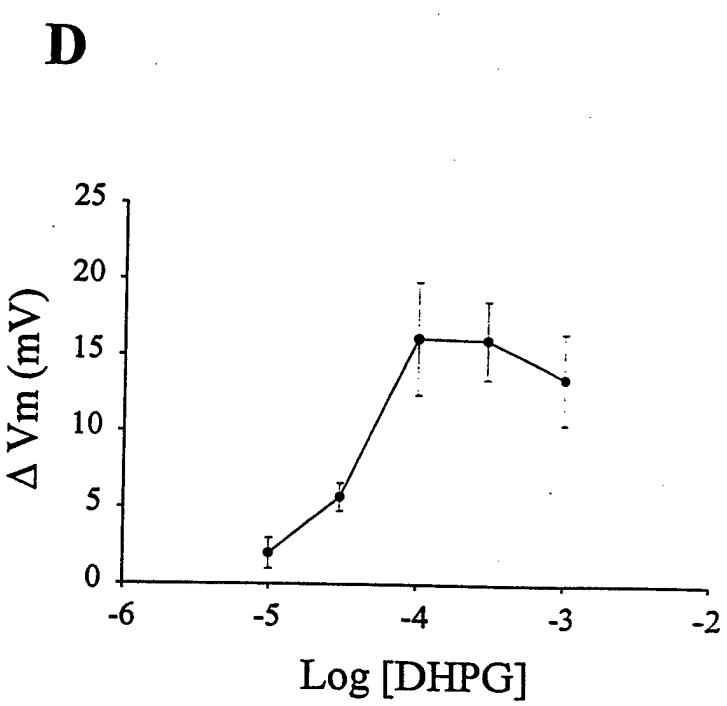


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Figure 3

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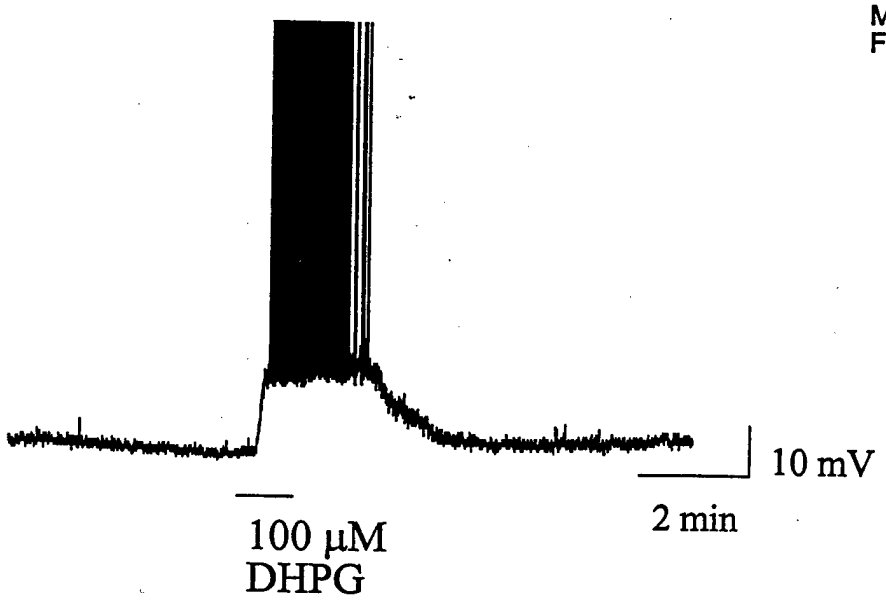

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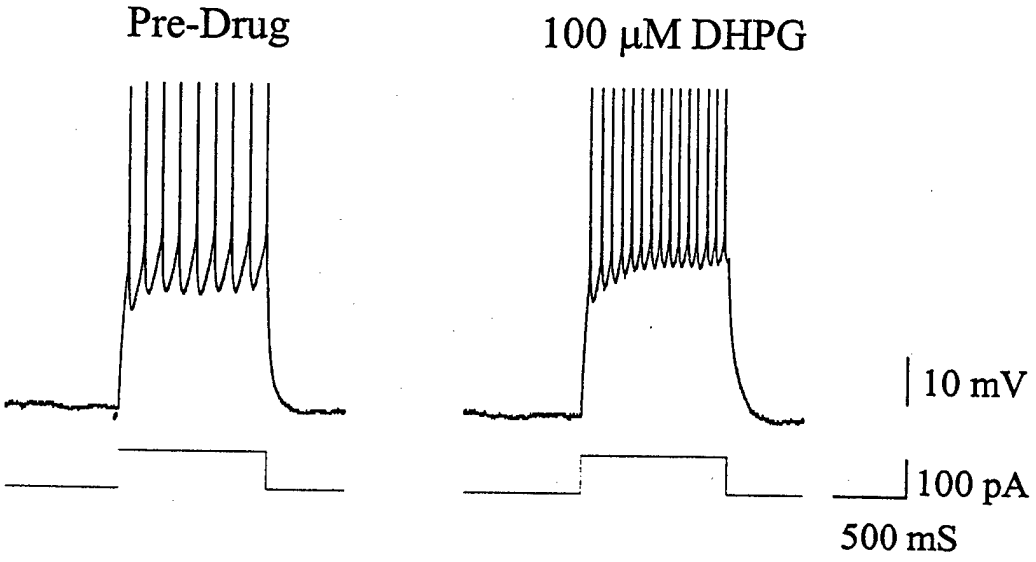

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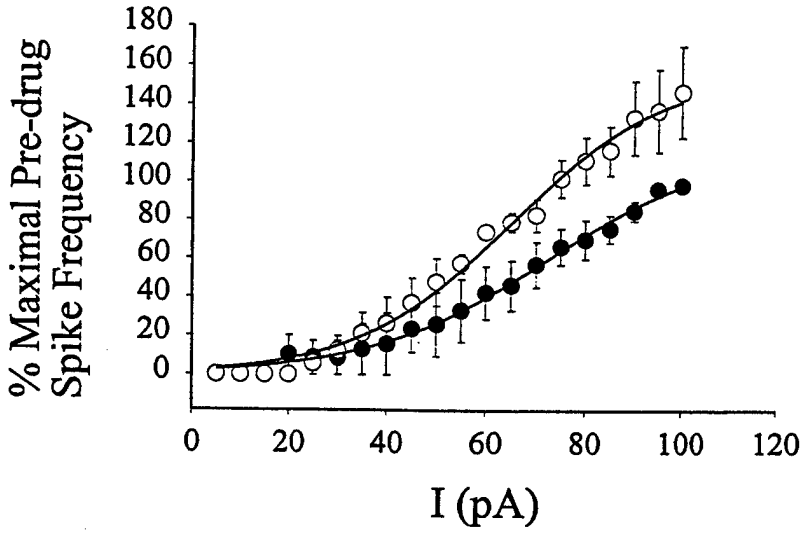

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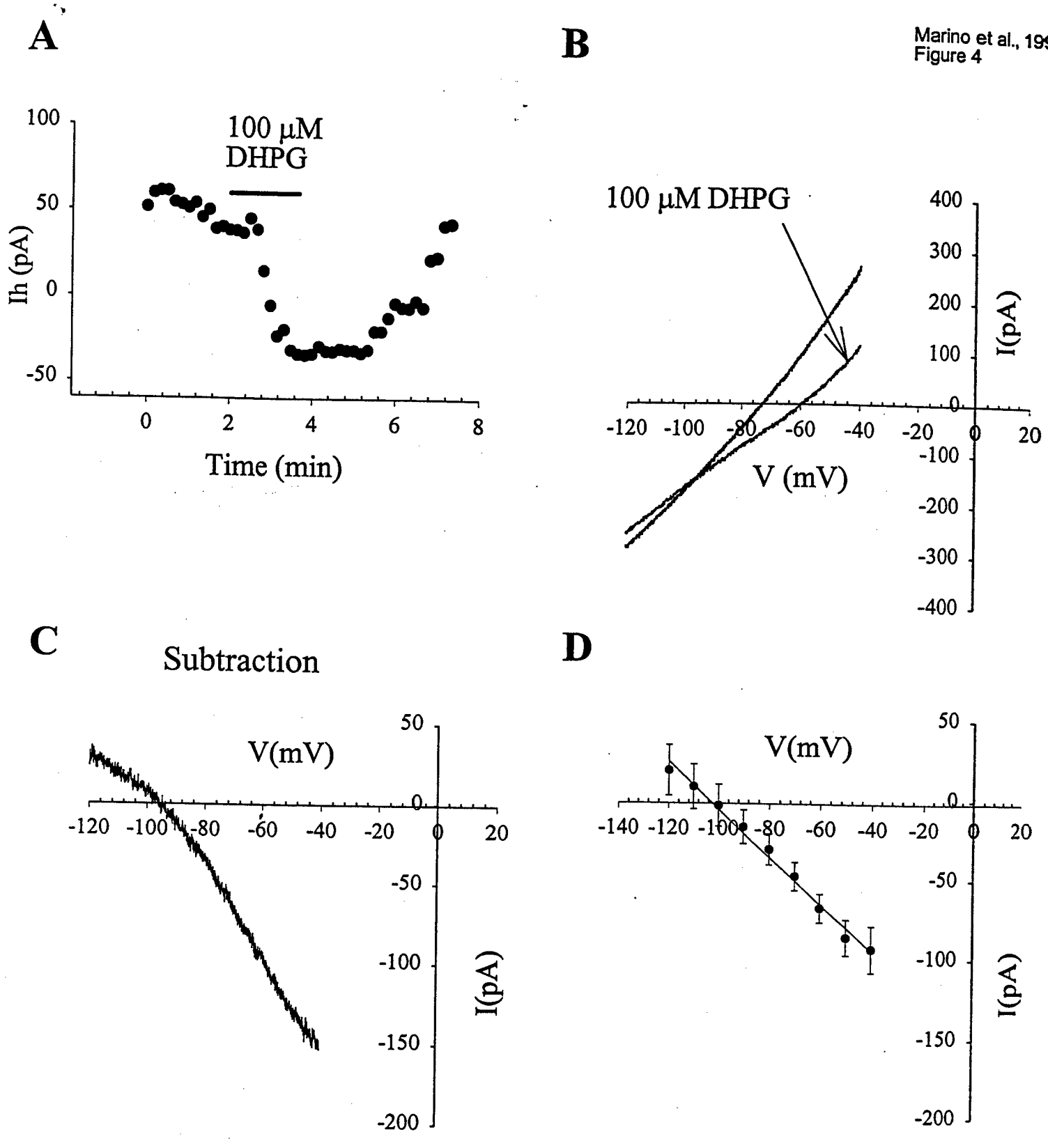
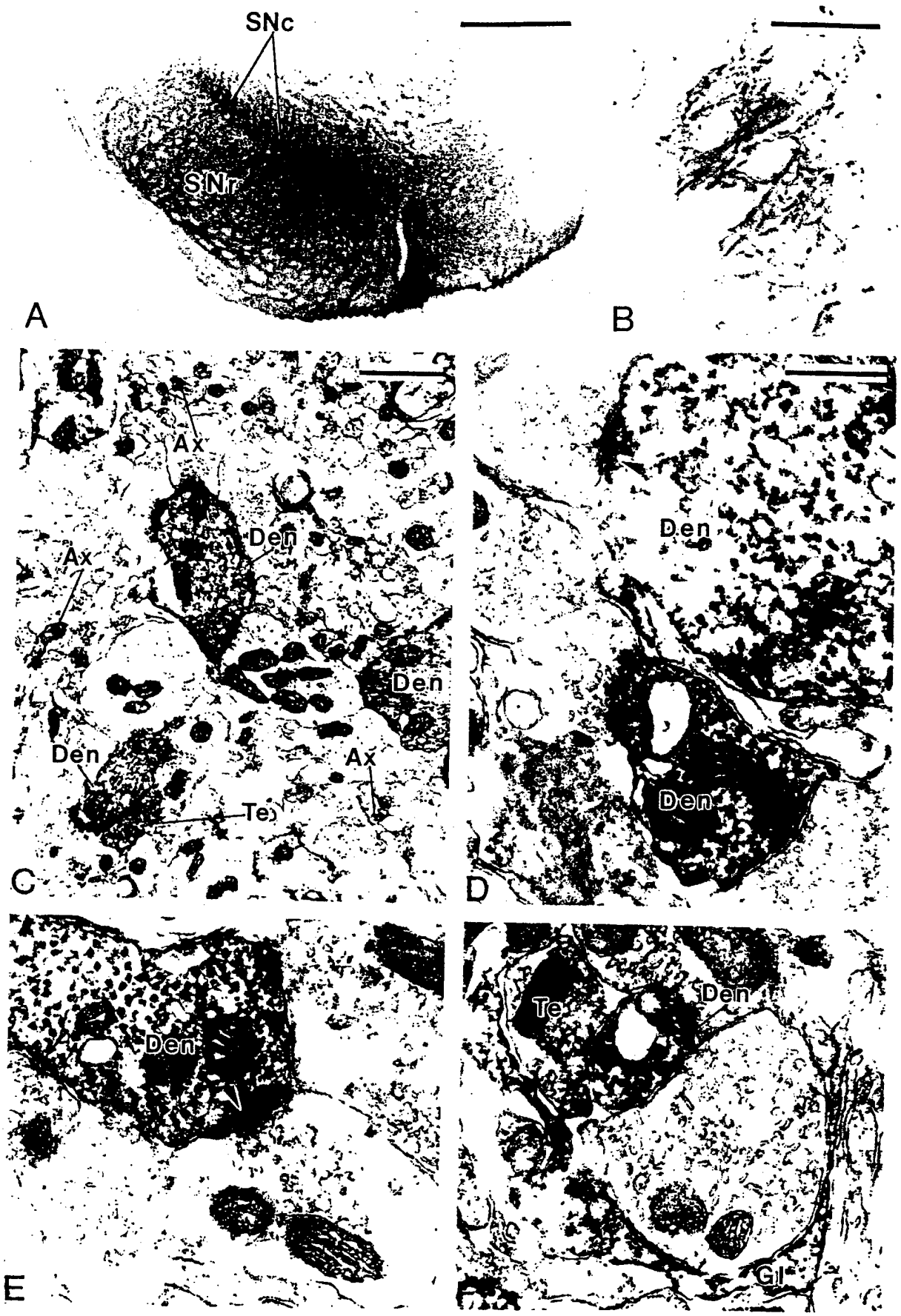
Figure 4

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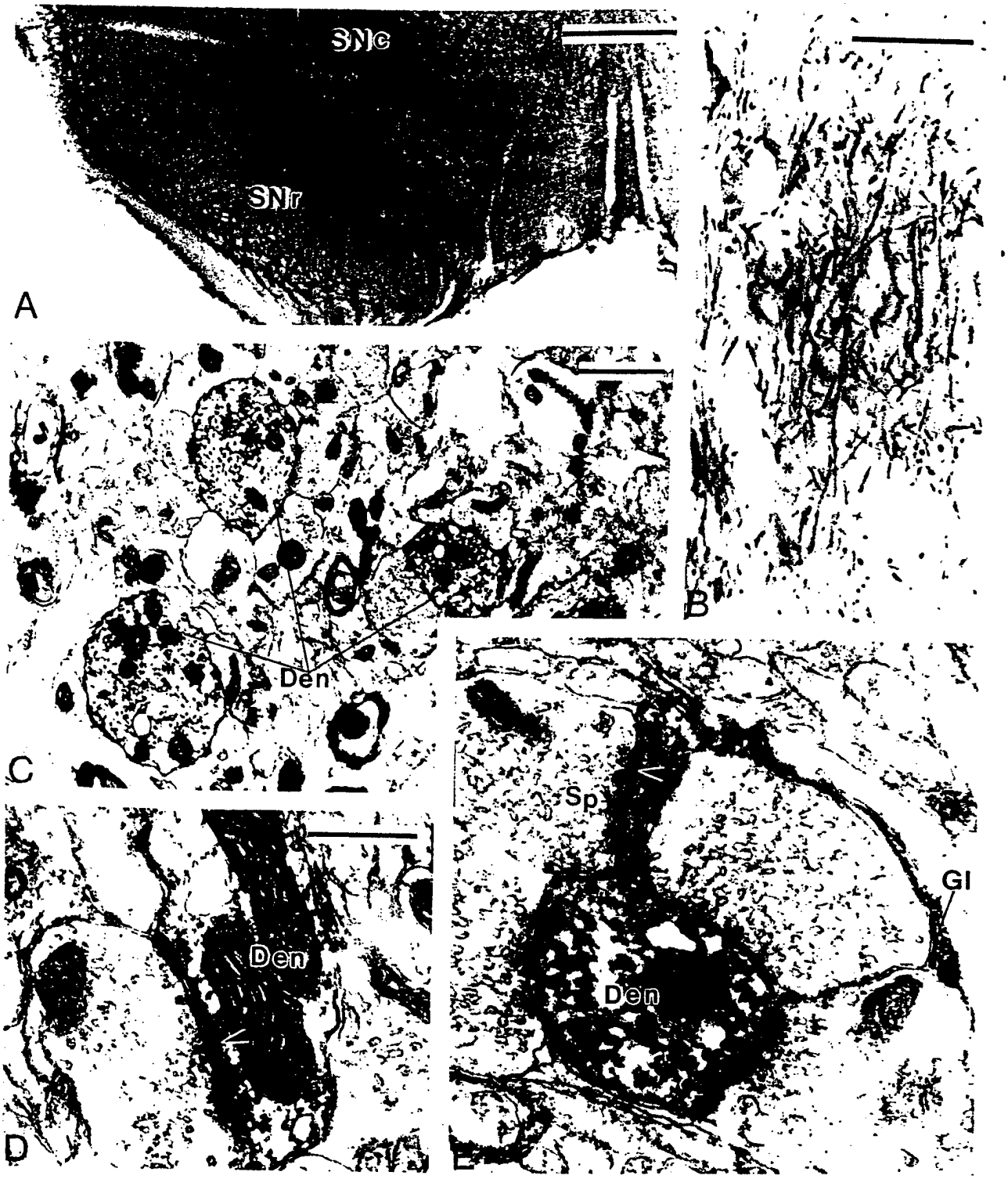
Marino et al., 1999
Figure 6



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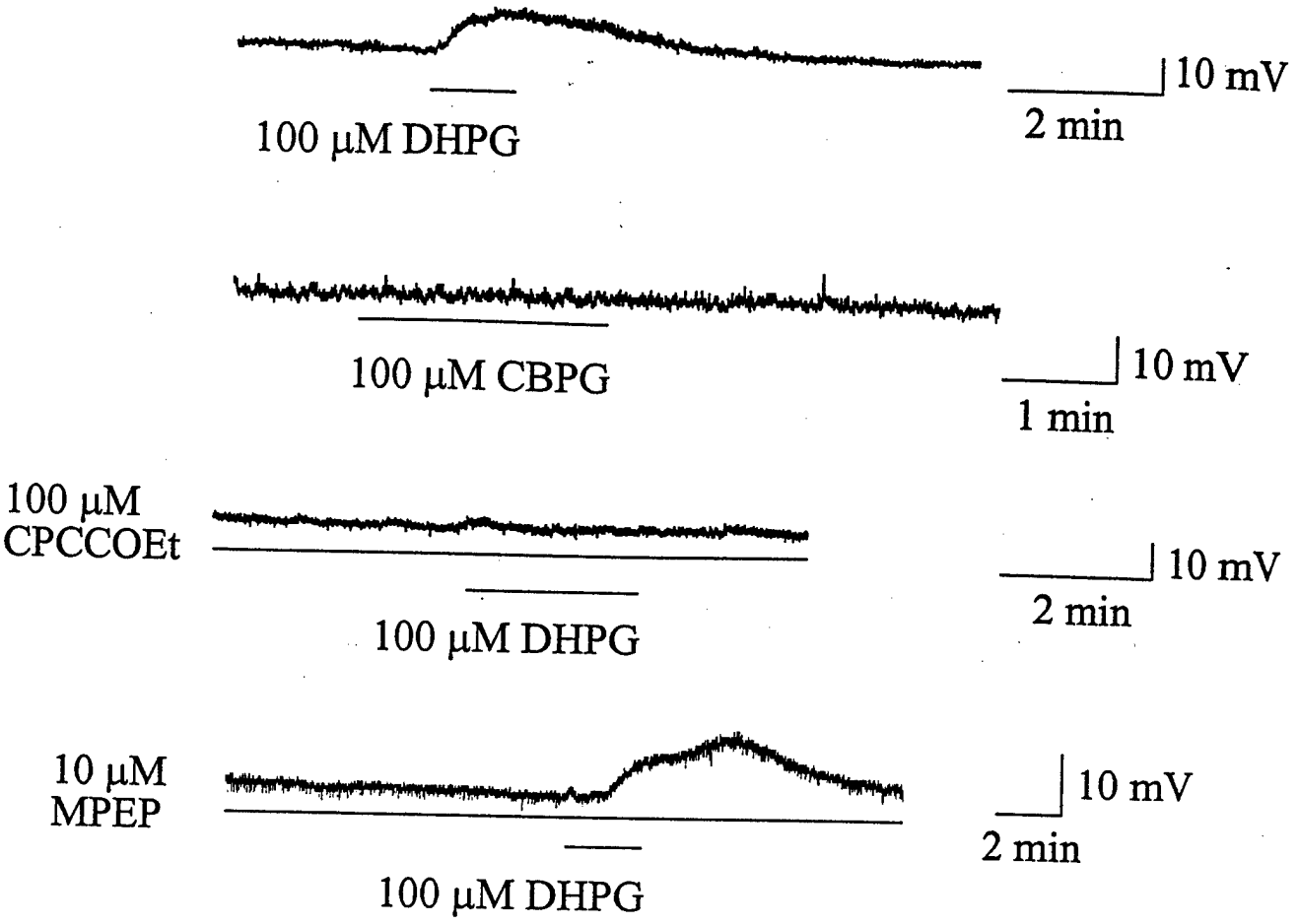
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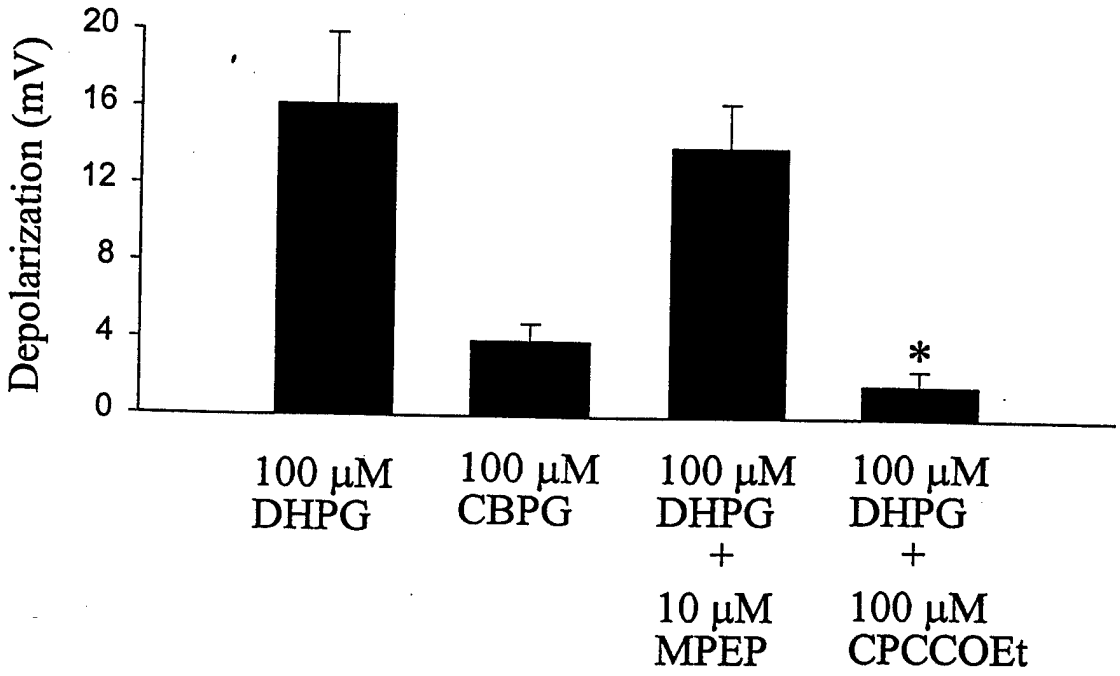
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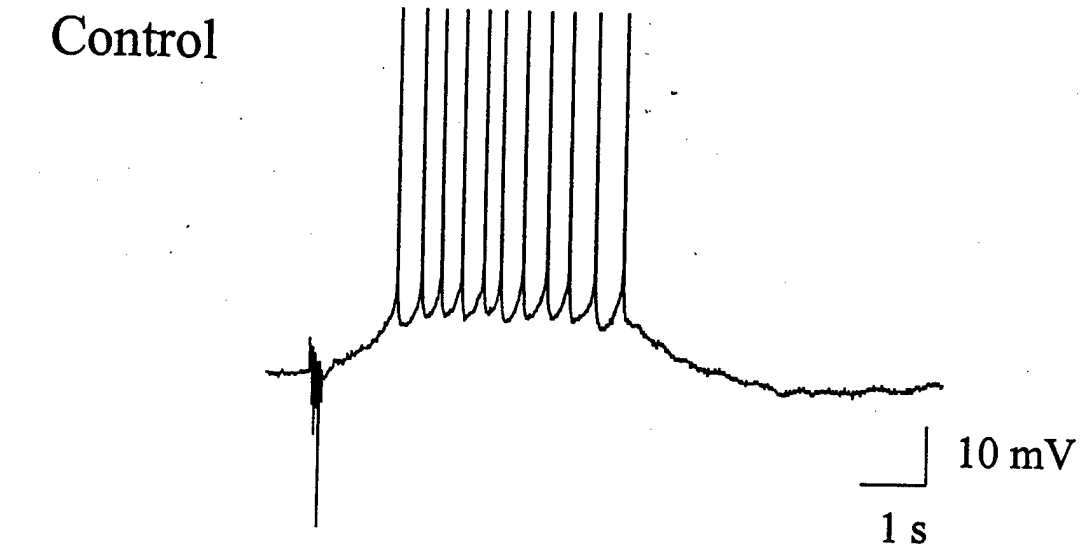
100 μM DHPG + 10 μM MPEP
100 μM DHPG + 100 μM CPCCOEt


Control
10 mV
1 s
100 μM CCPCOEt
Washout

176.15

REGULATION OF NEURONS OF THE SUBTHALAMIC NUCLEUS BY METABOTROPIC GLUTAMATE RECEPTORS. H. Awad*, P.J. Conn.
Department of Pharmacology, Emory University School of Medicine and Graduate Program in Molecular Therapeutics and Toxicology, Graduate Division of Biological and Biomedical Sciences, Atlanta, GA 30322.

Hyperactivity of neurons of the subthalamic nucleus (STN) is involved in the pathophysiology of various movement disorders, including Parkinson's disease. Agents that reduce excitation of the STN could have a therapeutic effect in the treatment of these disorders. Here we investigate the role of metabotropic glutamate receptors (mGluRs) in the regulation of STN neuron activity.

Electrophysiological recordings were made from STN neurons in horizontal rat brain slices. The group-I selective mGluR agonist dihydroxyphenylglycine (DHPG 100μM), but not the group II agonist (+)-2-aminobicyclo[3.1.0]hexane-2,6-dicarboxylate (LY354740 100nM) or the group III agonist L-2-amino-4-phosphonobutyrate (L-AP4 1mM), caused a direct depolarization of STN neurons (17.1±1.1mV). This group I-mediated depolarization was accompanied by a marked increase in cell firing and an increase in input resistance. The I-V curve shows a reversal potential of -80mV, consistent with a group I-mediated inhibition of a potassium channel. DHPG-mediated depolarization is significantly attenuated by the mGluR5 selective antagonist methylphenylethynylpyridine (MPEP 10μM; 4.2±0.3mV) but not the mGluR1 selective antagonist 7-hydroxyiminocyclopropan-[b]chromen-1a-carboxylic acid ethyl ester (CPCCOEt 100μM; 18.8±3mV). The mGluR5 selective agonist, (S)(+)-2-(3′-carboxybicyclo[1.1.1]-pentyl)-glycine (CBPG 100μM) mimicks the depolarizing effect of DHPG and is also blocked by MPEP but not CPCCOEt. These data suggest that mGluR5 mediates depolarization of STN neurons. We are currently investigating the effect of mGluR activation on ionotropic glutamate and GABA receptors in STN neurons and the role of mGluRs in modulation of transmission at excitatory and inhibitory synapses onto STN neurons. *(Supported by grants from NIH NINDS, and the U.S. Army).*

176.16

PHYSIOLOGICAL ROLES OF PRESYNAPTICALLY LOCALIZED TYPE 2,3 AND 7 METABOTROPIC GLUTAMATE RECEPTORS IN RAT BASAL GANGLIA

S. Risso Bradley[*,1], M.J. Marino[1], M. Wittmann[2], S. Rouse[1], A.I. Levey[3], and P. J. Conn[1], Depts of Pharmacology[1] and Neurology[3], Emory Univ. Sch. of Med. Atlanta, GA 30322, and Tierphysiologie[2], Univ. Tuebingen, D-72076 Tuebingen, Germany.

Metabotropic glutamate receptors (mGluRs) play a significant role in regulating basal ganglia (BG) function. BG are a set of interconnected subcortical nuclei that play a major role in the control of movement and pathophysiology of movement disorders. The input nucleus of the BG is the striatum, which receives innervation from the cortex. The primary output nuclei are the substantia nigra pars reticulata (SNpr) and the entopeduncular nucleus, which send inhibitory projections to the thalamus. The SNpr receives glutamatergic excitation from the subthalamic nucleus (STN). Hypokinetic movement disorders, such as Parkinson's disease (PD), in part result from an increased excitation of SNpr. Our immunocytochemistry studies at the electron microscopic level reveal that mGluR2/3 and mGluR7 are presynaptically localized in the SNpr on glutamatergic synapses. Therefore, we investigated the physiological roles of mGluR2/3 and 7 in rat slices of SNpr using the patch clamp technique in whole cell configuration. Our experiments suggest that activation of mGluR2/3 and mGluR7 inhibits excitatory transmission at the STN-SNpr synapse by a presynaptic mechanism. These data support the hypothesis that mGluR2/3 and mGluR7 may act as presynaptic receptors in the SNpr, where they play an important role in regulating glutamate release from STN-SNpr terminals. This provides a strong basis for development of novel therapeutic agents that target specific mGluR subtypes and could be used for treatment of PD and other disorders involving pathological changes in BG function. Supported by grants from NIH NINDS and U.S. Army.

176.17

DIRECT EXCITATION OF GABAERGIC PROJECTION NEURONS OF THE RAT SUBSTANTIA NIGRA PARS RETICULATA BY ACTIVATION OF THE MGLUR1 METABOTROPIC GLUTAMATE RECEPTOR. M.J. Marino*, S. Risso Bradley, M. Wittmann†, and P.J. Conn. Dept. of Pharmacology, Emory University, Atlanta, GA 30322 and, † Tierphysiologie, University of Tuebingen, D-72076 Tuebingen, Germany.

Behavioral and physiological studies have shown that metabotropic glutamate receptors (mGluRs) play important roles in regulation of basal ganglia (BG) function. Furthermore, specific mGluR subtypes are differentially localized throughout the BG. The predominant postsynaptic mGluRs are a group I mGluRs, while group II and III mGluRs are often localized presynaptically. This would indicate that group I receptors are localized in a manner consistent with direct modulation of the excitability of projection neurons. However, little is known about the physiological roles mGluRs play in regulating the function of BG structures. Here we demonstrate that activation of mGluRs by the group 1 mGluR selective agonist DHPG (30 μM - 1 mM) produces a direct depolarization of GABAergic projection neurons in the substantia nigra pars reticulata (SNr) along with a concomitant increase in input resistance. This effect is not mimicked by application of the group II - selective mGluR agonist LY354740 (100 nM) or by the group III-selective mGluR agonist L-AP4 (500 μM). Furthermore, the DHPG-induced depolarization is blocked by the mGluR1 subtype selective antagonist CPPCCOEt (100 μM), but is not blocked by the mGluR5 subtype selective antagonist MPEP (10 μM), and is not mimicked by the mGluR5 subtype selective agonist CBPG (100 μM). This provides strong evidence that the direct excitation of SNr projection neurons is mediated by mGluR1. Since the over excitation of SNr GABAergic neurons is believed to play a major role in the pathophysiology of Parkinson's disease, mGluR1 may provide an important target for new therapeutic agents that could be useful for treatment of this disorder. *[Supported by NIH NINDS and the U.S. Army]*

176.18

GABAERGIC INHIBITION OF RAT SUBSTANTIA NIGRA PARS RETICULATA PROJECTION NEURONS IS MODULATED BY METABOTROPIC GLUTAMATE RECEPTORS. M. Wittmann†, M.J. Marino, S. Risso Bradley, and P.J. Conn*. Depts of Pharmacology, Emory University, Atlanta, GA 30322 and† Tierphysiologie, University of Tuebingen, D-72076 Tuebingen, Germany.

The predominant inputs to the substantia nigra pars reticulata (SNr) are GABAergic projections from the striatum and the globus pallidus. Since over excitation of SNr output neurons is believed to play an important role in the pathophysiology of Parkinson's disease, the modulation of these GABAergic inputs may provide a crucial target for drug development.

Behavioral and physiological studies have shown that metabotropic glutamate receptors (mGluRs) play important roles in regulation of basal ganglia (BG) function. Furthermore, specific mGluR subtypes are differentially localized throughout the BG. The predominant postsynaptic mGluRs are a group I mGluRs, while group II and III mGluRs are often localized presynaptically. This would indicate that group II and III mGluRs are likely to play a role in presynaptic modulation of transmitter release. Here we examine the role of mGluRs in regulation of inhibitory synaptic transmission in the SNr. Application of the group I-selective agonist DHPG (100 μM) and the group III-selective agonist L-AP4 (500 μM) inhibit evoked IPSCs obtained by whole cell patch clamp recording from SNr GABAergic projection neurons. The group II-selective mGluR agonist LY354740 (100 nM) had no effect on the amplitude of the IPSCs. Interestingly, in other studies we have found that activation of group I mGluRs directly depolarize SNr GABAergic neurons. These findings suggest that activation of group I mGluRs can excite GABAergic projection neurons both by direct stimulation, and by disinhibition. Thus, group I mGluR antagonists could provide novel therapeutic targets for treatment of Parkinson's disease. Current work is aimed at more fully characterizing the pharmacology of this effect, and at determining the pre- or post-synaptic locus of this synaptic inhibition. *[Supported by NIH NINDS and the U.S. Army]*

sion in the DG, CA1 and CA3 regions 24 h after drug administration. These results support a role for VILIP-1 in the molecular changes which underlie persistant hippocampal LTP expression in vivo, and suggest that mGluR activation may function as a trigger for the upregulation in hippocampal VILIP-1 expression. VILIP-1 and other NCS-proteins, in turn, may lead to altered neuronal signaling during persistant long term potentiation.

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THE ROLE OF METABOTROPIC GLUTAMATE RECEPTORS IN PERSISTENT REDUCTIONS OF SYNAPTIC STRENGTH IN THE HIPPOCAMPUS OF FREELY MOVING RATS

D. Manahan-Vaughan. Institute for Physiology of the Charité, Department of Neurophysiology, Humboldt University, Tucholskystrasse 2, 10117 Berlin, GERMANY.

Metabotropic glutamate receptors (mGluRs) form a diverse group of G-protein coupled receptors which are localised in a relatively high concentration in the hippocampal formation. Information storage in the hippocampus is believed to require persistant, long-lasting alterations in the efficacy of synaptic communication. This may be expressed in the form of long-term potentiation (LTP), long-term depression (LTD) or depotentiation: forms of synaptic plasticity which are inducible in vitro, but also expressed in the hippocampus of freely moving rats. We have demonstrated that in freely moving rats, LTD and depotentiation are both induced by repetitive low-frequency stimulation, and comprise persistant and robust reductions in hippocampal synaptic efficacy. Pharmacological studies by this laboratory have further shown that whereas antagonists of group 1 mGluRs (e.g. (S)-4-carboxyphenylglycine) impair the expression of LTD or depotentiation in freely moving rats, antagonists of group 2 mGluRs mGluRs (e.g. 2S-alpha-ethylglutamic acid) demonstrate dose-dependent differential effects on these forms of synaptic plasticity. On the other hand, group 2 antagonists do not affect expression of LTP, whereas activation of group 1 mGluRs is critically required (Manahan-Vaughan, 1997; J.Neurosci. 17, 3293). These findings demonstrate, that in addition to mGluR involvement in LTP in vivo, mGluR activation is critically involved in the expression of forms of synaptic plasticity which involve persistant reductions of synaptic strength. Thus, mGluRs may function as bidirectional regulators of hippocampal synaptic plasticity. MGluR- regulated expression of synaptic plasticity in the hippocampus may be a key factor in the modulation of cellular mechanisms underlying learning and memory processes.

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SELECTIVE MODULATION OF SERTONIN2A RECEPTOR-MEDIATED GLUTAMATE EXCITATIONS IN THE RAT PREFRONTAL CORTEX BY GROUP II AND GROUP III mGlu RECEPTOR AGONISTS

G.J. Marek, Dept. of Psychiatry, Yale School of Medicine, New Haven, CT 06508.

Serotonin (5-HT), via 5-HT2A receptors, markedly increases spontaneous excitatory postsynaptic currents (spEPSCs) in the apical dendrites of neocortical layer V pyramidal cells, in part, by increasing glutamate release from a subset of presynaptic terminals (Aghajanian & Marek, 1997). The nonselective metabotropic glutamate (mGlu) agonist (1S,3S)-ACPD suppresses 5-HT-induced EPSCs by a presynaptic mechanism. Therefore, agonists selective for group II and III mGlu receptors were investigated for effects on 5-HT-induced spEPSCs and electrically-evoked EPSPs (evEPSPs; stimulation of subcortical fibers) in rat brain slices by intracellular recording from layer V pyramidal cells of the medial prefrontal cortex. The group II mGlu agonist LY354740 preferentially suppressed 5-HT-induced spEPSCs as compared to evEPSPs (EC_{50}'s = 89 vs 231 nM). The group II mGlu antagonist LY341495 potently blocked the suppressant effects of LY354740. Furthermore, the group II mGlu antagonist *increased* the frequency and amplitude of 5-HT-induced spEPSCs consistent with the hypothesis that mGlu2/3 acts as a presynaptic autoreceptor in this subpopulation of glutamatergic terminals. The group III metabotropic agonist L-SOP also suppressed 5-HT-induced spEPSCs (EC_{50} = 16 μM) but with only slight (< 10%) suppression of evEPSPs. These findings show that group II and III metabotropic agonists selectively suppress 5-HT-induced spEPSCs as compared to evEPSPs. Further data will be discussed suggesting that the 5-HT-induced spEPSCs reflect activity at thalamocortical synapses while the evEPSPs may predominantly involve activity at cortico-cortical synapses. Since most atypical antidepressant and antipsychotic drugs block 5-HT2A receptors, group II and/or III metabotropic agonists have a potentially promising therapeutic profile of action. Supported by NIMH and NARSAD.

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POTENTIAL ANTIPARKINSONIAN ACTIONS ON METABOTROPIC GLUTAMATE RECEPTORS IN THE SUBSTANTIA NIGRA PARS RETICULATE

M.J. Marino*, S.R. Bradley, M. Wittmann, S.T. Rouse, A.I. Levey and P.J. Conn. Depts. of Pharmacology, and Neurology Emory University, Atlanta, GA 30322 and, Tierphysiologie, University of Tuebingen, D-72076 Tuebingen, Germany.

In the rat, the major output nuclei of the basal ganglia (BG) are the substantia nigra pars reticulata (SNr) and the entopeduncular nucleus (EPN). Inhibitory projections from the striatum influence the activity of these output nuclei by direct, and indirect pathways. A fine tuning of motor control requires an intricate balance of activity in these pathways, and a disruption of this balance is believed to play a major role in the pathophysiology of Parkinson's disease (PD). Recent studies reveal that loss of nigrostriatal dopamine neurons in PD patients results in a dramatic increase in activity of the indirect pathway and increased firing of glutamatergic neurons in the STN. The increased excitation of GABAergic projection neurons in the output nuclei leads to the disabling motor impairment characteristic of PD. Metabotropic glutamate receptors (mGluRs) are often localized presynaptically on glutamatergic terminals where they can inhibit glutamate release. Interestingly, the group II mGluRs (mGluR2 and mGluR3) are expressed in STN neurons and these receptors have been shown to regulate glutamate release in other brain regions. This led us to postulate that group II mGluRs are presynaptically localized on STN terminals in the SNr and that activation of these receptors would reduce excitatory synaptic responses. Here we demonstrate that activation of presynaptically localized group II mGluRs inhibits excitatory transmission at the STN-SNr synapse. This suggests that a selective group II mGluR agonist could ameliorate the motor dysfunction associated with Parkinson's disease. Consistent with this, we find that the highly selective group II mGluR agonist LY354740 reverses catalepsy in an animal model of Parkinson's disease. [Supported by NIH NINDS and the U.S. Army].

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(2S, 1'S, 2'S, 3'R)-2-(2'-CARBOXY-3'-(3,3-DIPHENYLPROPYL) CYCLOPROPYLCLYCINE (UPF 665): A NEW SELECTIVE mGluR2 ANTAGONIST: SYNTHESIS, PRELIMINARY EVALUATION AND MOLECULAR MODELING STUDIES

Maura Marinozzi, Laura Amori, Roberto Pellicciari, Gabriele Costantino. Istituto di Chimica e Tecnologia del Farmaco, Via del Liceo, 1 I-06123 Perugia (Italy) Serge Bischoff, Peter Josef Flor.